

**ROLE OF CYCLOPHILINS AND HEAT SHOCK
PROTEINS IN MYOCARDIAL ISCHAEMIA /
REPERFUSION INJURY**

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**A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF
PHILOSOPHY IN THE UNIVERSITY OF LONDON**

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AKNOWLEDGEMENTS

First and foremost I am particularly indebted to Professor Colin Green for giving me the opportunity to do this thesis. I would like to thank my supervisors Dr Larisa Andreeva and Professor Barry Fuller for their consistent support, and Dr Richard Heads at The Rayne Institute, St Thomas's Hospital for his guidance and instruction in the techniques of molecular biology.

I would also like to thank everybody in Surgical Research who have helped me directly or indirectly in the completion of this project, in particular Padmini Sarathchandra, Mark Harrison, and Milan.

Many thanks go to my friends and family who in their own way have encouraged and supported me, especially Regna Ainsworth for the use of her computer. I am most grateful to my brother Waheed, without whom none of this would have been possible.

ABSTRACT

Restoration of blood flow after myocardial infarction (MI), surgery or fibrinolytic therapy is necessary, but can lead to cardiomyocyte dysfunction within a generalised condition commonly known as “reperfusion injury”. The role of cyclophilins, heat shock proteins (HSP), and the mitochondrial chaperone complex (MCC), was studied in this pathological condition.

In vitro and *in vivo* models were used to replicate conditions of ischaemia / reperfusion (IR) injury. H9c2 and COS-7 cell lines were employed in nitric oxide (NO) donor and transfection applications. Experimental protocols were used to determine mitochondrial membrane potential ($\Delta\psi_m$), mitochondrial morphology, protein expression, enzyme activity and cell damage in these models.

No difference was observed in activity or expression in cyclophilin or expression of the MCC in any of the models. It was noted that in the *in vitro* model, cell death was predominantly necrotic with only a minority of cells undergoing apoptosis, and as the degree of ischaemia increased cell death was 100% necrotic. The NO donor afforded protection against lethal ischaemia in H9c2 cells, but there was no detectable increase in expression of any HSP's. Transfection of cyclophilin-D (CyP-D, CyP3) into COS-7 cell lines demonstrated no changes in any tested parameters. Both subsarcolemmal (SSM) and interfibrillar (IFM) mitochondrial $\Delta\psi_m$ decreased in all experimental groups, which were reperfused after ischaemia. More importantly SSM $\Delta\psi_m$ decreased to a greater extent than IFM $\Delta\psi_m$, therefore demonstrating a greater sensitization to IR.

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LIST OF ABBREVIATIONS

AMP	adenosine monophosphate
ATP	adenosine 5' triphosphate
Ca^{2+}	calcium ion
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanine monophosphate
CO_2	carbon monoxide
Fe^{2+}	ferrous ion
Fe^{3+}	ferric ion
g	gram(s)
h	hour(s)
H^+	hydrogen ion
H_2O_2	hydrogen peroxide
i.m.	intramuscular
i.p.	intraperitoneal
i.v.	intravenous
l	litre
M	molar
min	minute(s)
ml	millilitre
mmol	millimole
mol	moles
MW	molecular weight
n	number in a group
Na^+	sodium ion
NADH	reduced nicotinamide adenine dinucleotide
NADP	oxidised nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
nm	nanomoles
NO	nitric oxide

OD	optical density
O ₂	oxygen gas
<i>P</i>	probability
pH	potential of hydrogen (acidity)
pO ₂	oxygen partial pressure
sec	seconds
μl	microlitre
μM	micromolar
vs.	versus
w/v	weight/volume

CHAPTER 1

1. INTRODUCTION TO CARDIOVASCULAR DISEASE

1.1 History of Cardiovascular Disease

Cardiovascular disease (CD) is a number one killer of both men and women in all ethnic groups in the UK and USA. This has been the situation since the 20th century. Before the 1900's very few people died of CD, because of poor hygiene many people died of infectious diseases such as: dysentery, cholera, typhoid, polio, plague and tuberculosis. (Although some of these infections may have resulted in some form of cardiovascular disease).¹ This was before the advent of the industrial revolution, when most people made their living through some sort of manual labour. The main form of transportation was walking, people washed their own clothes and many chores were carried out by hand. With the arrival of automation many of these tasks were taken over by machinery and therefore physical activity became unnecessary. Along with the change in lifestyle also came a change in diet, as high fat treats were easier to produce, which in all, made people more prone towards cardiovascular disease. Heart disease was rising so dramatically that it became commonplace especially during 1940 and 1967, which led to the World Health Organization describing it as one of the world's most serious epidemics.

1.2 Risk factors for Cardiovascular Disease

High levels of low-density lipoprotein (LDL) cholesterol and reduction in the levels of high-density lipoprotein (HDL) cholesterol are known to be risk factors in the development of heart disease. HDL is known as "good cholesterol" because it helps to remove "bad cholesterol" (LDL) from the blood and prevents it clogging up the arteries,

therefore reducing the risk of arteriosclerosis. Hypertension (high blood pressure) predisposes to left ventricular hypertrophy (LVH). LVH is described as the increase in the size of the individual muscle cells, generally visualised as thickening of the wall of the ventricle. Studies have revealed that LVH seriously increases the risk of cardiovascular disease.² It is considered to be a greater risk factor than cigarette smoking or hypertension.² Patients with diabetes mellitus often demonstrate a four-fold increase in propensity to suffer from a vascular disease than a non-diabetes patient and commonly show symptoms at a much younger age. The greater prevalence of obesity in the United States correlates with the increased risk to heart disease faced by Americans than other populations in the Western world. The increased morbidity due to obesity is mediated through metabolic consequences such as decreased HDL cholesterol and hypertension, but one of the most potent risk factors is visceral adipose tissue, which can be determined by waist circumference.

Nowadays there are many diagnostic modalities to help us identify the development of CD. These are positron emission tomography (PET), magnetic resonance imaging and electrocardiogram (ECG), which can be used to determine the viability of myocardial tissue and help the clinician to determine the optimum procedure to be used.

So what is CD? To explain what this is, it is better to explain the normal function of the heart (Figure 1-1). The heart is a small muscular organ located in the centre of the chest, which is composed of four chambers. From the upper chambers (atria), deoxygenated blood is carried from the venae cava to the right atrium of the heart into the lower chambers called ventricles, which with every heart beat pumps blood via the pulmonary artery (blue vessel) to the lungs. There the blood gets rid of its load of CO₂ and is replenished with oxygen. This oxygenated blood then enters the left atrium (red vessels) via the pulmonary vein and into the left ventricle whence it is pumped to all parts of the body via the aorta, which normally takes around a minute before the cycle continues again. A built in pacemaker called the sinoarterial node controls this cycle. The pacemaker functions by generating electrical signals that spread throughout the myocardium, triggering the heart to pump blood out.

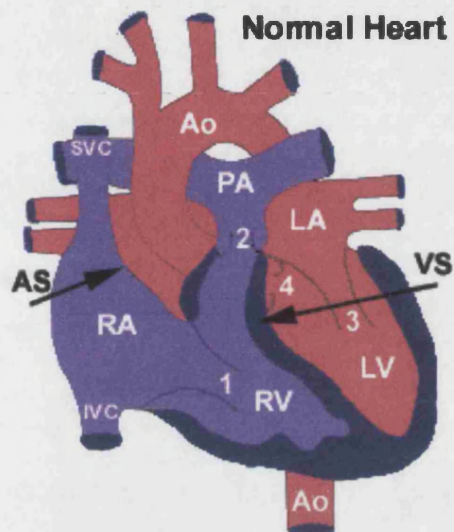


Figure 1-1. Diagram of the heart.

*LV:left ventricle, RV: right ventricle, LA: left atrium, RA: right atrium, LA: left atrium, AS: atrial septum, VS: ventricular l septum, 1:tricuspid valve, 2:pulmonary valve, 3:mitral valve, 4:Aortic valve, Ao:aorta, PA:pulmonary artery, SVC:superior vena cava, IVC:inferior vena cava

*Diagram taken from Royal Children's Hospital, website (www.rch.unimelb.edu.au/)

1.3 Heart Disease

This is a broad term referring to many types of cardiovascular disorders, which come under the umbrella term heart disease. The heart like any other muscle requires a continuous supply of oxygen and nutrients, which is transported to the heart by blood in the coronary arteries. This is prevented in the condition called atherosclerosis.

1.3.1 Atherosclerosis

This is a disease, which leads to the formation of fibrous plaques (fatty deposits) on the inner surface of artery walls that along with the proliferation of smooth muscle cells and accumulation of lipids, results in occlusion of the artery. When atherosclerosis is severe (>60%), there is a major reduction in the supply of oxygenated blood to the heart. This results in symptoms characterized by severe chest pain called angina.

1.3.2 Angina Pectoris

Angina pectoris “angina” is best described as recurring pain localised to the chest area. This occurs when the blood supply to the heart is restricted such as in atherosclerosis. Angina occurs during emotional stress, consumption of alcohol and physical exercise. These are common triggers for angina because they are conditions where the heart demands a greater supply of blood. Angina is symptomatic of coronary heart disease and if left untreated, will soon result in myocardial infarction.

1.3.3 Hypertension

Hypertension or “high blood pressure” often called the “silent killer” because by the time people find they have this condition, it is often too late. The reason for this is that

the symptoms are not easily detectable and consequently most people who have this condition are quite unaware.

With each heartbeat, blood pressure is generated by blood being forced through the small constricting blood vessels of the circulatory system and in this manner oxygen, nutrients and other materials are supplied to the cells. As the demand for these nutrients changes, the circulatory system responds by constricting or dilating the arteries, which alters the blood pressure and therefore the speed at which the blood travels. In some people these arteries remain constricted and thereby cause the blood pressure to remain high. This increase in blood pressure as well as damaging the circulatory system also damages the brain, kidney and the eyes.

1.3.4 Myocardial Infarction

Myocardial infarction (MI) is the correct term for a heart attack. This term means that as a result of the lack of supply of oxygen and other nutrients to the heart cells, these cells die. As a clinical entity, MI has been redefined by a document produced at a conference by the joint European Society of Cardiology/American College of Cardiology Committee for redefinition of myocardial infarction² as “myocardial cell death due to prolonged ischaemia”.

1.3.5 Current Procedures Used to Rectify Cardiovascular Conditions.

These are angioplasty as well as the use of drugs.

1.3.6 Percutaneous Transluminal Coronary Angioplasty (PTCA)

Balloon angioplasty or PTCA is a commonly used non-surgical procedure in blockages of the coronary artery. A small incision in the groin or arm is needed to insert a small catheter to keep the artery open. Attached to the catheter is a deflated balloon, which is inflated in order to compress the blockage and thereby provide an adequate passage for blood to flow. If successful, PTCA can reduce the risk of heart disease and may prevent the patient needing to undergo the more invasive coronary artery bypass surgery. This procedure is quite simple and may take from 20 minutes to a few hours. In arteries that are totally occluded, or have bends, PTCA is not recommended. PTCA may actually weaken the artery such that a heart attack may result and sometimes restenosis may occur in previously PTCA treated arteries.

If PTCA is not successful in all situations previously described or where there are multiple occluded arteries, then a common surgical treatment called coronary artery bypass surgery or grafting (CABG) is used.

1.3.7 Coronary Artery Bypass Grafting (CABG)

This procedure utilises pieces of veins or arteries from the legs, which are then connected to the arteries of the heart in order to bypass the blocked vessels and thus increase the flow of blood to the heart. This procedure lasts several hours and the patient is placed in intensive care immediately after the operation, probably leaving hospital 3 – 7 days later if the procedure is successful.

In an occluded artery, an injection or intravenous drip can be used to administer a thrombolytic agent such as streptokinase. These thrombolytic agents boost the action of an enzyme called plasmin, which dissolves the clot and restores normal blood flow. These drugs are administered after a patient has a heart attack, which in some cases can be between 4 to 24 hours afterwards. Many clinical trials have now demonstrated³ that

these thrombolytic drugs are most effective within 90 minutes post MI in combination with the use of aspirin.

There are generally four types of medications used to treat CD: anti-platelet drugs, nitrates, calcium channel antagonists and beta-blockers. Aspirin is a drug that interferes with platelet aggregation and therefore the clotting process. It makes platelets less sticky and thus prevents clots forming on fatty plaques, which block the artery. It is because of this action of thinning the blood that makes it effective in reducing a person's risk for heart disease.

Nitrates in the form of nitroglycerin can give immediate relief from the pain of angina, when the heart muscle is not getting enough oxygen. As oxygen is supplied to the heart muscle via blood, then the blood supply must be increased. Nitrates such as nitroglycerin dilates the blood vessels and so give relieve to angina. Administration is in the form of an oral spray, tablet, patch or paste. Short acting nitrates last for 30 minutes but longer acting nitrates are available.

Other types of medication used are beta-blockers, which are able to reduce the heart rate by blocking cardiac β -receptors. The neuroendocrine system governs catecholamines, which are a group of hormones that include norepinephrine. When released into the blood, catecholamines increase the heart rate by acting on the β -adrenergic receptors. Investigators have determined that patients with chronic heart disease had increased norepinephrine plasma levels. These levels were normal in milder cases of the disease; therefore abnormally high levels are indicative of a poor prognosis.⁴

1.4 Ischaemia /Reperfusion Injury

1.4.1 Cell Injury During Ischaemia

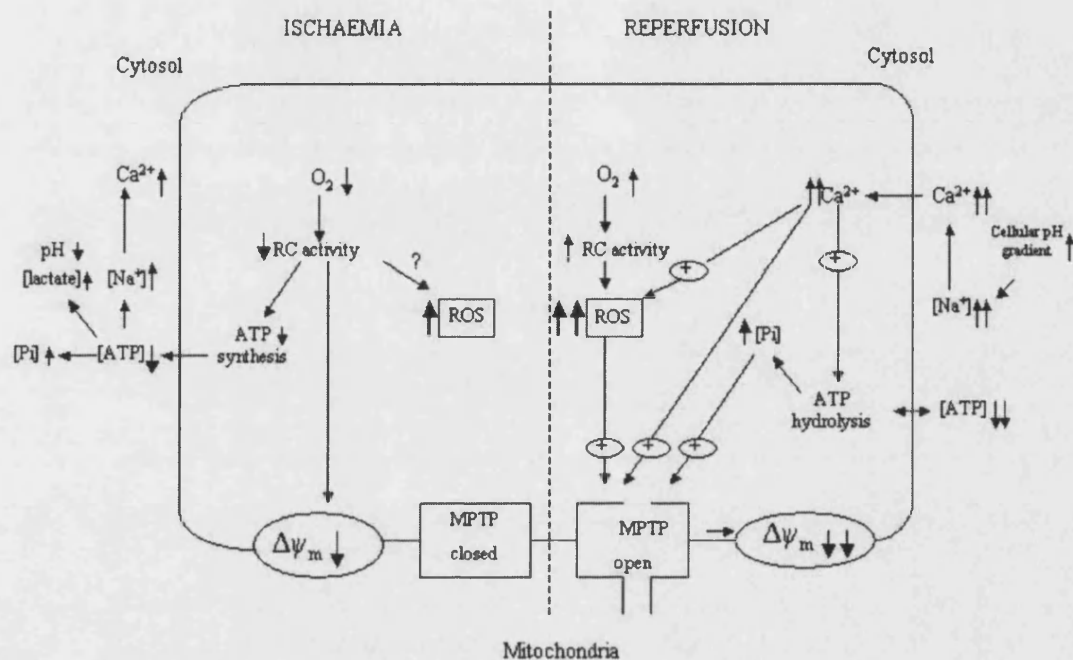


Figure 1-2. This is a schematic of the movement of ions during ischaemia/reperfusion.

As ischaemia ensues, adenosine triphosphate (ATP) synthesis is halted as respiratory chain (RC) activity slows down and ATP levels drop, inorganic phosphate levels increase (P_i). Anaerobic glycolysis is switched on in order to maintain the proton motive force (pmf) and the resultant production of lactate reduces matrix pH below ~ 7 which prevents the MPTP from opening (pH paradox). During reperfusion, matrix pH increases, RC activity is restored and ATP synthesis continues, but sudden influx of O_2 results in increased production of ROS and therefore oxidative stress Ca^{2+} overload. If there is not sufficient ADP and ATP and antioxidants such as glutathione, then these conditions promote the opening of the MPTP with a concomitant decrease in mitochondrial membrane potential ($\Delta\psi_m$) eventually resulting in inhibition of mitochondrial function and cell death. (Diagram from Morin and colleagues)⁵

In myocardial cells, upon obstruction of blood flow, oxidative phosphorylation is unable to produce the energy rich adenosine 5'-triphosphate (ATP) and so these levels decrease and pH decreases as metabolism switches from aerobic oxidation to anaerobic glycolysis producing lactic acid (Figure 1-2). This is quickly followed by a reduction in contractile force, as the energy-dependent pumps in the cellular membranes are impaired. In an attempt to restore the pH, the Na^+/H^+ antiporter is activated and as ATP levels are low and the Na/KATPase is inhibited which, with the increase of cytosolic Na^+ , the Na^+/H^+ antiporter does not pump Na^+ out. This results in an increase in the level of Ca^{2+} as the $\text{Na}^+/\text{Ca}^{2+}$ antiporter is inhibited and as a consequence, does not pump Ca^{2+} out resulting in a decrease in $\Delta\psi_m$. The increased Ca^{2+} intracellular levels result in the release of enzymes such as protease, phospholipases and nucleases, which, depending on the duration of ischaemia may cause permanent damage to tissue. Mitochondrial respiration decreases and as a result of adenine nucleotide catabolism, hypoxanthine accumulates which will become toxic upon the re-introduction of blood flow. At this point damage is reversible if the blood flow is re-established but, if ischaemia progresses, then cardiomyocyte membrane integrity is reduced, thereby resulting in ultrastructural changes and cell death is inevitable. Usually it takes about 15 minutes of myocardial ischaemia before cell death begins to appear ^{2,6} in the myocardium.

1.4.2 Reperfusion

Following cardiac therapies, reperfusion of ischaemic tissue is fundamental in order to salvage cardiomyocytes and in some cases, damage caused by myocardial ischaemia, can be reversed. The patient in the condition previously described as angina may often experience transient periods of ischaemia, but due to immediate restoration of blood flow, permanent damage is not usually incurred.

1.4.3 Reperfusion/ Injury

Although reperfusion of ischaemic tissue is necessary, it may paradoxically cause more harm than good especially in cases where ischaemia has been long and severe. Then upon reperfusion, any cardiomyocyte damage sustained during the preceding ischaemia is exacerbated, resulting in a predisposition to a pathology of conditions called ischaemia /reperfusion (IR) injury.⁷

Myocardial stunning is an established manifestation of IR injury. It is defined as “myocardial dysfunction that persists after reperfusion despite the absence of irreversible damage”.⁸ This contractile dysfunction is completely reversible, but requires a long period of time before the myocardium regains total functionality. The use of inotropic agents helps to reverse this syndrome.

Hibernation is another manifestation of IR injury although it helps to avoid an energy deficit, in the context of coronary heart disease, maintaining myocardial integrity and viability,⁹ and is defined as “ a form of prolonged contractile dysfunction associated with ongoing low blood flow”.¹⁰ It is thought that hibernation may represent chronic stunning, as repeated ischaemic episodes causes prolonged myocardial stunning which lasts longer than the stunning exhibited after a single ischaemic bout.¹¹ Some authors suggest from their experimental data in humans, that different biochemical mechanisms are involved in the pathophysiology of stunning and hibernation.¹² However, it has been demonstrated in pigs, that repetitive stunning induces myocardial hibernation.¹³ Myocardial hibernation and chronic stunning would seem to be phenomena that are very similar; however in the former case, blood flow is reduced, and in the latter, blood flow is normal.¹¹ Clinical evidence has led to speculation, that hibernation is an adaptation process, because in times of reduced coronary flow and energy availability, reduction in contractile activity is an appropriate response. Ultrastructurally de-differentiation changes have been observed leading to the postulation that this is a degenerative process.¹⁴ Little is known about myocardial stunning and less about hibernation. However, it is believed that the key players in the pathogenesis of myocardial stunning are reactive oxygen species (ROS) and Ca^{2+} .^{6,11,15} In view of this,

during the 1980's two hypotheses were proposed which are the oxyradical hypothesis and the calcium hypothesis. The first postulates that ROS is involved whilst the calcium hypothesis postulates that disruption of Ca^{2+} homeostasis is a causative factor in myocardial stunning. Both are related to each other so their separate roles cannot be clearly defined in the pathophysiological process of myocardial stunning.^{11,16}

1.4.4 *ROS and Ca^{2+} as the Main Mediators of IR Damage*

ROS may be oxygen-centered radicals that contain one, or more unpaired electrons, for example, superoxide anions (O_2^-) and hydroxyl radicals (OH^\cdot) or covalent molecules such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl). Some ROS, as they contain unpaired electrons, are better described, as free radicals of which O_2^- and OH^\cdot are good examples.^{17,18} These ROS cause oxidative stress, occurring as the generation of ROS increases, overwhelming endogenous antioxidant defence mechanisms leading to the oxidation of biological molecules. Examples include deoxyribonucleic acid modification and the more common lipid peroxidation damage of polyunsaturated fatty acid contained in cell membranes.^{19,20} ROS can also stimulate leukocyte activation, leukocyte adhesion molecules and cytokine gene expression.⁸

The main sources of ROS are hypoxanthine and the mitochondrial respiratory chain. An enzyme xanthine oxidase converts hypoxanthine to xanthine in the presence of oxygen, producing oxygen free radicals as a by-product of this reaction. Generally in non-ischaemic cells, hypoxanthine is converted to uric acid by xanthine dehydrogenase, a reaction that does not produce ROS. During ischaemia, increased Ca^{2+} concentrations initiate the conversion of xanthine dehydrogenase to the oxidase form, xanthine oxidase.¹⁶ In the other source of ROS (mitochondrial respiratory chain), the respiratory chain is inhibited by the lack of oxygen and consequently ubiquinone is reduced to ubisemiquinone. This upon reperfusion transfers an electron to molecular oxygen to produce H_2O_2 which is toxic, but is not a free radical; however, in the presence of heavy metals (Fe^{2+} or Cu^{2+}) it is converted into the more toxic OH^\cdot species.^{8,17,19,21} Reperfusion also serves to attract and activate neutrophils that also produce O_2^- free

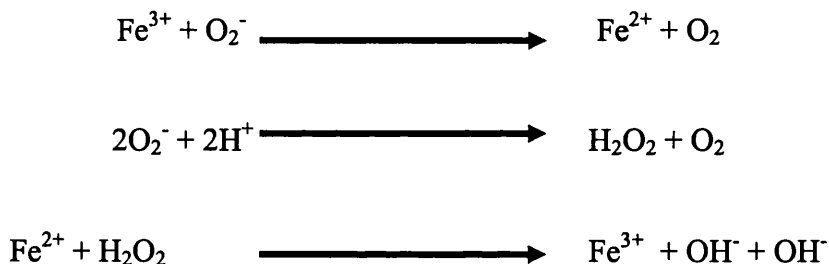
radicals through the membrane bound NADPH oxidase system thereby causing further oxidative injury.^{6,17}

The superoxide ion (O_2^-) is a weak oxidising agent and is incapable of peroxidising membrane lipids. The injury that occurs is dependent on more powerful oxidising agents such as OH^\cdot . Generally in the presence of H_2O_2 and superoxide generating systems, the Haber –Weiss equation, produces OH^\cdot .¹⁷



Equation 1-1. Haber-Weiss

Without a catalyst this reaction proceeds too slowly; however, in the presence of transition metals, the reaction speeds up. During ischaemia as the pH drops, iron (Fe) attached to an Fe carrier protein in plasma (transferrin), starts to dissociate making it available for catalysing formation of OH^\cdot in the Fenton reaction. Fe^{2+} is deemed to be the most significant metal catalyst in physiological systems.¹⁷



Equation 1-2. Fenton reaction

In this reaction Fe^{3+} is acting as a redox agent and accepts an electron from oxygen in the manner of a Fenton catalyst.

Many investigators have demonstrated that oxidative stress increases upon reperfusion. The ROS species identified include H_2O_2 , O_2^- , OH^\cdot and peroxynitrate. This manifests itself in the heart as arrhythmias, loss of adrenergic pathways, changes in gene expression and a reduction in contractile function. These clinical results have been

paralleled with experimental systems containing ROS generating substances. The increase of ROS during ischaemia remains controversial.²² Vanden Hoek and colleagues observed that significant amounts of O_2^- and H_2O_2 were generated during ischaemia as well as during reperfusion.²²

Increased intracellular Ca^{2+} levels are associated with IR induced cardiomyocyte damage. It is now widely accepted that the balance between influx and efflux of Ca^{2+} across the mitochondrial inner membrane regulates mitochondrial dehydrogenases, which are rate-limiting for citric acid cycle metabolism.²³ In short, when cell activity leads to a substantial or excessive rise in Ca^{2+} , the mitochondria may accumulate large amounts of calcium.²⁴ This is due to the uniporter carrying calcium into the mitochondrion along the electrochemical gradient, which has a much higher total activity than the export pathways, which encompass Na^+ - Ca^{2+} exchange. This basically means that if the net influx of Ca^{2+} exceeds the capacity of the extrusion pathway through Na^+ - Ca^{2+} exchange, intramitochondrial Ca^{2+} concentration increases, and Ca^{2+} will be sequestered within the mitochondria. This is detrimental to the mitochondrion and may lead to the formation of a large conductance pore called the mitochondrial permeability transition pore (MPTP).²⁵ MPTP formation results in cessation of mitochondrial function which is an underlying cause of necrotic and apoptotic cell death.²⁶⁻²⁹ However, it is believed that the MPTP may participate as a Ca^{2+} release channel when calcium overload occurs.²⁴ The increased Ca^{2+} encourages the production of O_2^- and this in combination with the available Fe drives the Fenton chemistry production of OH^- .¹⁹ The increasing levels of Ca^{2+} also result in activation of calcium-dependent proteases leading to myofibril proteolysis.⁶

Mitochondria as well as being one of the main sites of ATP synthesis, is also one of the main production sites and targets of oxidative damage. With ROS species emanating from the respiratory chain of the mitochondria, as part of normal cell respiration.^{30,31} In mitochondria, organic compounds are oxidised by atmospheric oxygen to CO_2 and H_2O ³²(Figure 1-3) and the energy produced is stored as ATP (Figure 1-3). Oxidation of NADH supplies electrons to complex I of the respiratory chain which reduces a

membrane associated ubiquinol/ubiquinone (UQH₂/UQ) pool via complex II. Oxidation of flavin-linked dehydrogenases reduce UQH₂/UQ as they do not possess a negative enough redox potential to enter complex I. Electrons are transferred from ubiquinol to complex III and then to cytochrome C (Cyt C), which in turn reduces complex IV (cytochrome oxidase). From complex IV, 4 electrons are transferred to molecular oxygen. In this circuit, the complexes I, III and IV are essentially acting as proton pumps in respect to the electron flux. As the electrons pass through these complexes the redox potential decrease is used to generate a proton electrochemical potential gradient ($\Delta\mu_{H^+}$), which is generally expressed in electrical potential units as (Δp) the proton motive force (pmf) and is represented by the following equation.

$$\Delta p \text{ (mv)} = \Delta\psi_m - (2.3RT/F) \Delta pH \quad (\text{At } 37^\circ\text{C } \Delta p = \Delta\psi_m - 60\Delta pH)$$

Equation 1-3. Generation of the proton motive force (pmf)

ΔpH = pH gradient across the inner membrane where a positive value is indicative of an acidic matrix.

R, T and F refer to the gas constant, absolute temperature and the Faraday constant

As demonstrated above (Equation 1-3), the pmf is composed of the ΔpH and the mitochondrial membrane component ($\Delta\psi_m$) of which under most conditions $\Delta\psi_m$ is the dominant component.

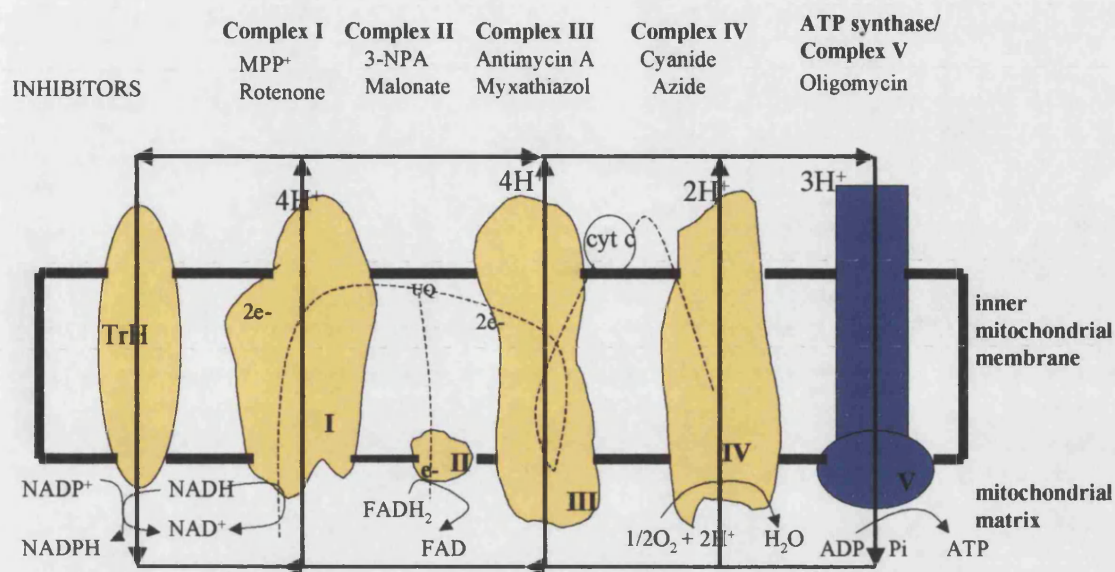


Figure 1-3. Schematic of the chemiosmotic proton circuit.

Electrons deriving from intermediary metabolism enter the respiratory chain at complexes I and II. These electrons are transferred by the respiratory chain by reduction and oxidation of enzyme complexes to oxygen with the generation of water via a process linked to H⁺ ejection generating an electrochemical gradient known as the proton motive force (pmf). The generation of the pmf is then used to drive ATP synthesis. Also listed are the range of respiratory chain inhibitors such as rotenone, which by blocking electron transfer at specific sites, block respiration through the whole chain. This will cause hydrolysis of ATP, but at a rate such that the function of the cell is not compromised, as long as glycolysis is still able to maintain sufficient ATP^{25,32} Diagram adapted from review by Nicholls and Budd.³²

When ATP is split into adenosine diphosphate (ADP) and inorganic phosphate (Pi), a large amount of energy is liberated which can be used to drive many cellular processes. ATP is commonly known as the universal energy carrier within the cell. The actual process where ADP and inorganic phosphate (Pi) form ATP from respiration is termed “oxidative phosphorylation”. The mechanism by which electron transfer coupled to ATP is synthesised during oxidative phosphorylation was not known until the 1960’s. Many researchers such as Chance, Slater, Lehninger and Ernster performed in-depth pioneering work on mitochondrial research during this time, but it was Peter Mitchell who formulated an alternative view on the coupling of electron transfer to ATP synthesis. He proposed that the flow of electrons through the respiratory chain drives positively charged hydrogen ions or protons across the mitochondrial membranes that

generate an electrochemical potential gradient across the membranes. This gradient is composed of: a difference in electric potential and a difference in hydrogen ion potential, or pH, which Mitchell collectively calls the “protonmotive force”. ATP synthesis occurs by the reverse movement of electrons down this gradient (Figure 1-3). Mitchell’s proposal was called the “chemiosmotic theory”³³, that is now a fundamental principle in bioenergetics and resulted in him being awarded the Nobel prize for Chemistry in 1978.^{32,34,35}

Mitochondrial energy production may also be harnessed in different ways, diverted from the business of ATP production into the generation of heat through the expression of catecholamine-regulated uncoupling proteins in brown fat in neonates and in hibernating mammals.³⁶ It has also been suggested that these proteins may be evolved in regulating the production of free radical species. This is particularly useful in the scenario where electrons from the fatty acid chain are fed to the electron transport chain, in excess to that which is required to produce ATP. This would then result in the overproduction of ROS³⁴ possibly leading to the pathological opening of the mitochondrial permeability transition pore (MPTP). Increased levels of these uncoupling proteins reduce ROS levels and therefore the formation of MPTP.³⁴

Elevated levels of ROS in combination with high levels of Ca^{2+} , adenine nucleotide depletion and increased inorganic phosphate accumulation, induce opening of a Ca^{2+} dependent reversible permeabilisation of a non-specific, high conductance pore in the mitochondrial inner membrane.^{29,37} This pore is commonly known as the mitochondrial permeability transition pore (MPTP).^{38,39} This process is termed mitochondrial permeability transition (MPT) originally described by Hunter and colleagues.⁴⁰ Mitochondrial Ca^{2+} overload is not the only major causative feature of MPT, as Ca^{2+} overload alone appears to be relatively harmless since ATP concentrations remain high and the cell may still be viable. This situation changes with the addition of the other factors symptomatic of IR such as depletion of adenine nucleotides and high phosphate concentrations.⁴¹ Also it has been determined that ROS via oxidation of critical thiol groups contributes to the formation of the MPTP.⁴² The formation of the MPTP allows molecules of <1500 Daltons to travel across this channel resulting in mitochondrial

membrane potential ($\Delta\psi_m$) depolarisation due to a reduction of the proton motive force (pmf). Therefore $\Delta\psi_m$ is indicative of the energy status of the mitochondrion and therefore of homeostasis in general.^{25,32,43} The $\Delta\psi_m$ collapses after severe exhaustion of glycolytic ATP and/or oxidative damage. ATP depletion occurs as oxidative phosphorylation breaks down and calcium overload leads to mitochondrial swelling and outer membrane rupture.^{41,44} Mitochondria control cell death pathways by determining whether the mode of cell death is by apoptotic or necrotic pathways. This is believed to be dependent on the intracellular ATP levels.⁴⁵ Transient opening means that ATP levels may be maintained, as apoptosis is an energy requiring process resulting in the release of pro-apoptotic factors such as apoptosis inducing factor (AIF) and cytochrome c.⁴⁶ It has been suggested that transient opening could imply a role for the pore in calcium homeostasis.⁴⁷ During extensive opening of the pore ATP production stops, ionic gradients cannot be maintained and with the release of degradative enzymes the damage becomes irreversible and therefore necrosis ensues.^{41,48,49} At this point mitochondria switch from being the major ATP producers to being the major ATP consumers due to the hydrolytic action of ATPase as the mitochondria struggles to maintain the pmf.^{44,48}

The main protein composition of the MPTP is controversial, but it is thought to be composed of the voltage dependent anion channel (VDAC) found in the outer membrane, cyclophilin D (CyP-D) located in the matrix, adenine nucleotide translocase (ANT) and associated kinases such as hexokinase and creatine kinase.⁵⁰ ANT is a specific carrier for ATP/ADP and is located in the mitochondrial inner membrane.⁵¹ The mechanism involved in the formation of the pore has been studied at length using agonists and antagonists of MPTP such as cyclosporin A, bongkreikic acid and carboxyatracyloside.

The ANT exists in 2 states “c” (cytoplasmic) and “m” (inner membrane) or “high” and “low” affinity states. When occupied by transportable substrates, the ADP/ATP-binding sites are either on the c- site or m-site.⁴⁹ Carboxyatracyloside when bound to the “c” state activates the pore whereas bongkreikic acid upon binding to the “m” state inhibits pore opening.⁴¹ In other words the c-state conformation is required for MPTP

formation. The immunosuppressive drug cyclosporin A binds to CyP-D⁵² and inhibits its binding to ANT thus preventing formation of the pore. VDAC and the ANT have recently been described as forming a complex, which straddles the inner and outer membranes,^{41,53} although Halestrap and colleagues⁵⁴ do not recognise the existence of VDAC in pore formation. They proposed that increasing Ca^{2+} levels causes the ANT to undergo a conformational change to form the pore. This process is greatly enhanced by the binding of CyP-D to the ANT and its binding increases with increasing oxidative stress. CyP-D possess peptidyl-prolyl cis-trans isomerase activity which when bound to the ANT is believed to induce a conformational change to form the MPTP.⁵⁵ A previous study demonstrated that when ANT-1, an ANT isoform, was overexpressed, this resulted in apoptosis. To explain this theory a model was proposed in which normal circumstances ANT-1 did not form the MPTP, due to the presence of inhibitors or endogenous repressors of pore formation. However, upon overexpression of ANT-1 titrated out these endogenous repressors to promote apoptosis. When CyP-D was co-expressed it suppressed the ANT-1 induced pore.⁵⁶

Griffiths and Halestrap developed a model to prove the existence of the mitochondrial pore opening during IR. They used $[\text{H}^3]$ -2-Deoxyglucose (DOG), which after entering the heart was metabolised to DOG-6-Phosphate; this, after being trapped in the cells will only enter the mitochondria once the pore forms. They ascertained that the pore did not open during ischaemia but 2 minutes post reperfusion.⁵⁷

1.4.5 Endogenous protection against ROS

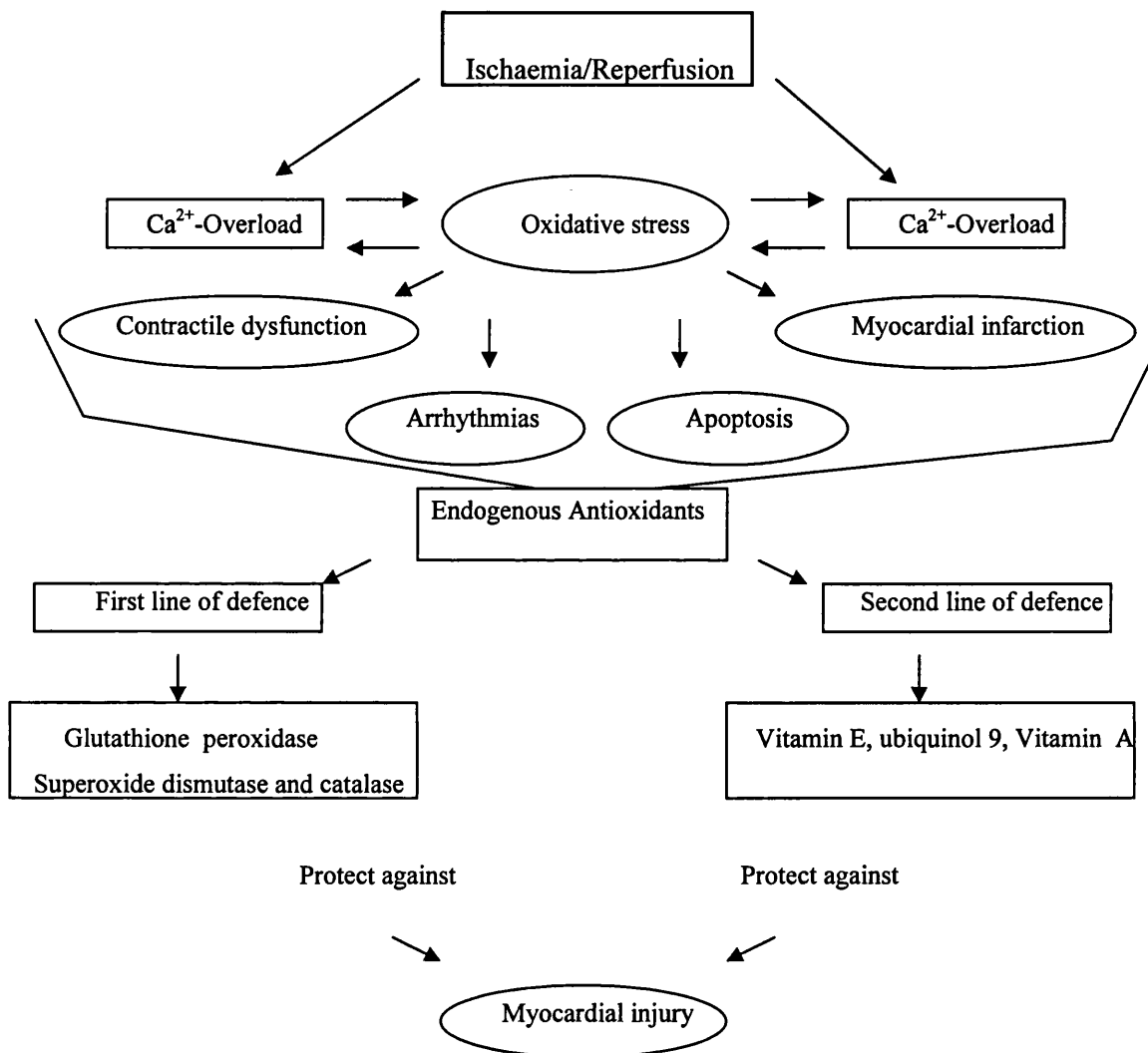


Figure 1-4. Ischaemia/reperfusion flow chart

Schematic diagram, detailing the main protagonists in ischaemia/ reperfusion injury and the manifestations of this phenomenon. Also mentioned are the various substances which inhibit and delay against ischaemia/reperfusion injury. (Modified from Dhalla and colleagues)¹⁶

The myocardium contains numerous endogenous protective systems that are activated upon reperfusion, such as the antioxidants. Antioxidants in the myocardium are substances that can delay or inhibit oxidative stress damage to carbohydrates, subcellular proteins, DNA and lipids. Even though the interactions and mechanisms in

different antioxidants are not completely understood, the possibility exists for one antioxidant to equilibrate with another and thereby establish a redox potential such that all antioxidants may act simultaneously to protect against an oxidative insult.¹⁶ It has been speculated that by different mechanisms, antioxidants can: (1) prevent the production of ROS as well as scavenge them and their precursors; (2) increase endogenous antioxidant formation; (3) attenuate ROS production via binding to metal ions; and (4) induction of the anti-death gene (BCl-2) thus reducing apoptosis.¹⁶

Table 1-1. Endogenous antioxidants

A list of the major endogenous antioxidants, which are found in cardiomyocytes (taken from Dhalla and colleagues 2000)¹⁶

Name	Site	Action
Superoxide dismutase (SOD)	-	Catalyses O_2^- dismutation to H_2O_2
Cu, Zn SOD	Cytoplasm, cell	$2O_2 + 2H^+ \rightarrow H_2O_2 + O_2$
	Surface and	
Mn SOD	mitochondria	
Catalase	Peroxisomes and	$H_2O_2 \rightarrow 2H_2O + O_2$
	mitochondrial membrane	
Glutathione peroxidase	Cytoplasm	$H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG$
Glutathione	Intracellular	Cellular reductant
Coenzyme Q10	Cell membrane	Redox active electron
(ubiquinone)		carrier
Vitamin E (α -tocopherol)	Cytoplasm and	Break lipid peroxidation
	plasma	chain and LDL reaction
β -Carotene (pro-vitamin A)	Plasma	Inhibits oxidation of LDL
Vitamin C (ascorbic acid)	Cytoplasm and	Directly as an antioxidant or
	plasma	as a cofactor for vitamin E

Exogenous antioxidants are drugs containing thiol compounds such as β -adrenergic blockers, iron chelating agents and Ca^{2+} antagonists. Examples of known endogenous antioxidants are shown in Table 1-1.

Glutathione (GSH), catalase and SOD have been extensively studied. GSH is the most important and is an intracellular non-protein sulphhydryl antioxidant, which is depleted during ischaemia. Its primary role is in the maintenance of cellular proteins and lipids in a functional state, protecting them against the threat of oxidation. Other roles include: a) regeneration of ascorbate (AA) from dehydroascorbate (DHA), its oxidised form; 2) detoxification of hydrogen peroxide and other toxic intermediates of drug metabolism and lipid peroxides. Further reductions in the levels of these antioxidants increase oxidative stress.¹⁶

Antioxidant levels have been found to decrease to varying degrees depending on the severity of IR injury. Ascorbate and glutathione are hydrophilic substances whose levels decrease after 40 minutes of reperfusion but not following ischaemia.¹⁶ Contrary to this, ubiquinol 9 and vitamin E are lipophilic antioxidants and their levels are unchanged during IR. Moreover, the addition of H₂O₂ increases the severity of IR resulting in the depletion of vitamin E and ubiquinol 9. This suggests that ascorbate and glutathione are the first line of defence against IR whereas vitamin E and ubiquinol 9 are considered to be the second line (Figure 1.4).

Once it was recognised that ROS played a major role in reperfusion injury, attention turned to mechanisms to attenuate their harmful effects. This led to a widespread interest in antioxidant therapy.⁶ Classical models of ischaemia/reperfusion have given mixed results, due to the variety of experimental protocols. However clinical results have been quite disappointing according to some investigators.⁶

1.4.6 Heat Shock Proteins

Heat shock proteins (HSP) are synthesised when an organism is subjected to environmental or physiological stresses. They are classified according to their molecular weight in kilodaltons (kDA). These proteins are members of large multigene families, which are present in all major cellular compartments.⁵⁸

Stress disrupts protein homeostasis resulting in the appearance of non-native proteins, which if left, are prone to misfold or aggregate causing cellular damage. Heat shock proteins restore protein homeostasis and in this manner confer cytoprotection against stress-induced damage. This is one of the reasons why many HSP's are referred to as molecular chaperones.⁵⁸

1.4.7 Molecular Chaperones

Molecular chaperones are proteins that assist the folding, refolding, translocation and transport of proteins. They provide a stable environment for non-native proteins to achieve the native state without the risk of misfolding or aggregating.⁵⁹ One of the best studied is HSP70, which is upregulated in IR and its increasing expression is directly correlated with its cytoprotective properties.⁶⁰ It has been reported to be overexpressed in rat myogenic cell lines and rat hearts by many scientists and found to improve myocardial function.⁶¹⁻⁶³ Synthesis of heat shock proteins in response to stress is regulated by the heat shock transcription factor (HSF)^{58,64,65}.

Originally the idea was proposed that in the non-stressed cell, HSF1 is attached to HSP70. However, upon the appearance of non-native, misfolded or aggregating proteins, HSF1 detaches from HSP70,⁶⁵ trimerises and travels to the nucleus where it binds to the heat shock element (HSE) upstream of the heat shock promoter gene and initiates synthesis of various molecular chaperones including HSP70. New data indicate that HSP90 is bound to HSF1 and unbinding of HSP90 results in activation of HSF1 leading ultimately to HSP70 synthesis.⁶⁶

Newly synthesised polypeptides can fold spontaneously, or with the assistance of molecular chaperones such as HSP70, they fold to achieve the tertiary native form.⁶⁷ HSP70 binds to the exposed hydrophobic residues, preventing off-pathway reactions and transports those polypeptides destined to go to the mitochondria where its targeting sequence is recognised prior to the polypeptide being released and directed towards the

general insertion pore (GIP). The non-native protein passes through the GIP and along the inner membrane; this translocation procedure requires the interaction between mitochondrial HSP70 (mHSP70/HSP75) and Tim44,⁶⁸ which pulls the protein through and prevents it from sliding back. mHSP70 passes the protein to the chaperonin complex, which mediates folding to the native state. The chaperonins HSP60/HSP10, which are the mammalian homologues of the bacterial GroEL-GroES, are arranged in two rings in which the two six membered rings formed by groEL form a central cavity where protein folding takes place in the hydrophobic interfaces. The two ends of the central cavity are capped by groES.⁶⁹ It has been demonstrated that simultaneous overexpression of both components of the chaperonin complex (HSP60/HSP10) is necessary for the survival of myogenic cells after IR injury.⁶⁴

1.4.8 Preconditioning

Subjecting the whole heart or cardiomyocyte to an initial sublethal stress (commonly referred to as “pre-treatment”), is enough to enhance the tolerance of the heart or cardiomyocyte and render them relatively resistant to subsequent exposure to a more lethal stress. Using heat to precondition a cell will ensure that it is protected against other stresses (cross tolerance) such as UV light, heavy metals and amino acid analogues. This phenomenon is known as stress tolerance or preconditioning.⁷⁰

Adaptation of tissue to repeated stresses such as hypertrophy has long been appreciated, it was however, not commonly known that cells could revert back to their original phenotypic steady state.⁷¹ True preconditioning or ischaemic preconditioning (IPC) was first reported in 1986.⁷² When Murray and colleagues experimented in a dog's heart, they demonstrated in this canine model that hearts subjected to 40 minutes ischaemia, the size of the infarct was substantially reduced in preconditioned hearts as opposed to non-preconditioned. The authors concluded that this phenomenon was initiated by ischaemia, especially as the observed protection could not be explained by increased blood flow through collaterals.⁷² This phenomenon was later demonstrated to occur in

many species.⁷³ There is also evidence that ischaemic preconditioning exists in several organs such as the central nervous system⁷⁴ skeletal muscle,⁷⁵ intestine⁷⁶ and liver.⁷⁷ It was a few years later that Yellon and colleagues⁷⁸ suggested that it was possible to precondition human myocardium.

It is important to note that ischaemic preconditioning only delays the development of cell death. If the subsequent long lasting ischaemic period is prolonged to several hours, eventually all the cells will die.^{72,79} Furthermore, the protective effect of IPC is of limited duration.⁷⁹ Preconditioning occurs in two protective phases, the first classical or early and the second delayed or late. Murray and co-workers first described the early phase in 1986.⁷² Marber and colleagues,⁸⁰ who referred to it as the “second window of protection”, first described this phenomenon. Early preconditioning appears minutes after the initial stimulus, and lasts a short time, whereas delayed preconditioning requires longer to develop, but lasts for hours. Classical preconditioning has been demonstrated to protect against infarction and arrhythmias while the late stage in addition protects against myocardial stunning.⁸¹ The prolonged duration of protection makes delayed protection a viable option in the clinical setting. Short episodes of occlusion of one artery can protect against a sustained period of occlusion in another artery, a process known as remote preconditioning.^{82,83} Other authors have reported on more intriguing examples whereby preconditioning the small intestine and kidney in rats afforded protection to the myocardium, and their results suggested that the mechanism of protection involved activation of a neurogenic pathway.⁸⁴

The cellular mechanisms contributing to this endogenous protection system have been studied in great detail, in order to allow therapeutic induction of protection.⁸⁵ Even with the use of many *in vitro* and *in vivo* model systems, they still remain incompletely resolved. It has been proposed that initiation of signalling pathways require one or several “triggers”, which in turn activate one or a few “mediator(s)” and eventually activating one or several “end-effectors” as demonstrated in Figure 1-6.⁷⁹ These signalling pathways result in amplification of the initiating signal(s), and permit interaction between the different biochemical components. Pharmacological agents that can also mimic preconditioning, can block these intracellular pathways at certain

steps.⁸⁶ The classical and delayed form of preconditioning may have similar biochemical components, which may be activated at different times during the ischaemic episodes to trigger and/or mediate preconditioning thus offering differing degrees of protection.^{87,88} One of the first major breakthroughs was the discovery that preconditioning was receptor mediated.⁸⁹ Numerous agonists are released during ischaemia in particularly adenosine from the breakdown of ATP. Administration of adenosine antagonists blocked the IPC response whereas an infusion of adenosine afforded protection that was similar to IPC.⁸⁹ Hence adenosine was recognised as playing a critical role in triggering IPC. Parratt and colleagues⁹⁰ proposed that bradykinin is a major trigger in the anti-arrhythmic effect of ischaemic preconditioning. However, between different species, triggers of classical preconditioning may vary, for example. Adenosine is not involved in preconditioning the rat heart⁹¹ but is involved in the IPC of rabbit and human myocardium.⁸⁶ It was proposed by Goto and colleagues,⁹² that in order to trigger preconditioning, a threshold must be reached. This is characteristic of an “all or nothing” response equivalent to that seen in synaptic nerve transmissions. Whereby an accumulation of impulses is achieved until the threshold is reached thereby inducing synaptic transmission. It should be noted that preconditioning triggers are often different from mediators. Bradykinin is a good example of a trigger of IPC, but it is not important in the sustained ischaemic episode. In contrast adenosine, is an important trigger and mediator of preconditioning.^{79,88}

Some of the above mentioned triggers (Noradrenaline, Bradykinin and adenosine) are coupled to G protein and pre-treatment with pertussis toxin has been proven to block the protective effect of IPC.⁹³ It has been proposed that the activated receptors couple through G proteins to phospholipase C and the formed diacylglycerol activates protein kinase C and facilitates the translocation of the PKC to different subcellular compartments, a hypothesis proposed by Downey and colleagues⁹⁴ (Figure1-5). Experiments with the tyrosine kinase inhibitor genistein imply that PKC is not the only kinase involved in classic preconditioning. Researchers wondered if other substances released during ischaemia, such as epinephrine, acetylcholine and opioid-peptides, could also be involved in triggering the ischaemic preconditioning response by binding to inhibitory G-coupled receptors.⁹⁵ In addition, endogenous substances (angiotension

II, NO, O_2^- radicals, metabolic products, endothelin and Ca^{2+}) were also proposed to be involved in this phenomenon. Therefore IPC appears to involve activation of several signalling pathways. Integral members of these pathways are Norepinephrine, K_{ATP} channels, adenosine ⁹⁶ and protein kinase C. They participate in the signalling that occurs in delayed preconditioning. ⁷¹ This is also generally believed to involve manganese superoxide (MnSOD), HSP's, inducible nitric oxide synthase (iNOS) and cyclo-oxygenase-2 (COX-2). ⁹⁷

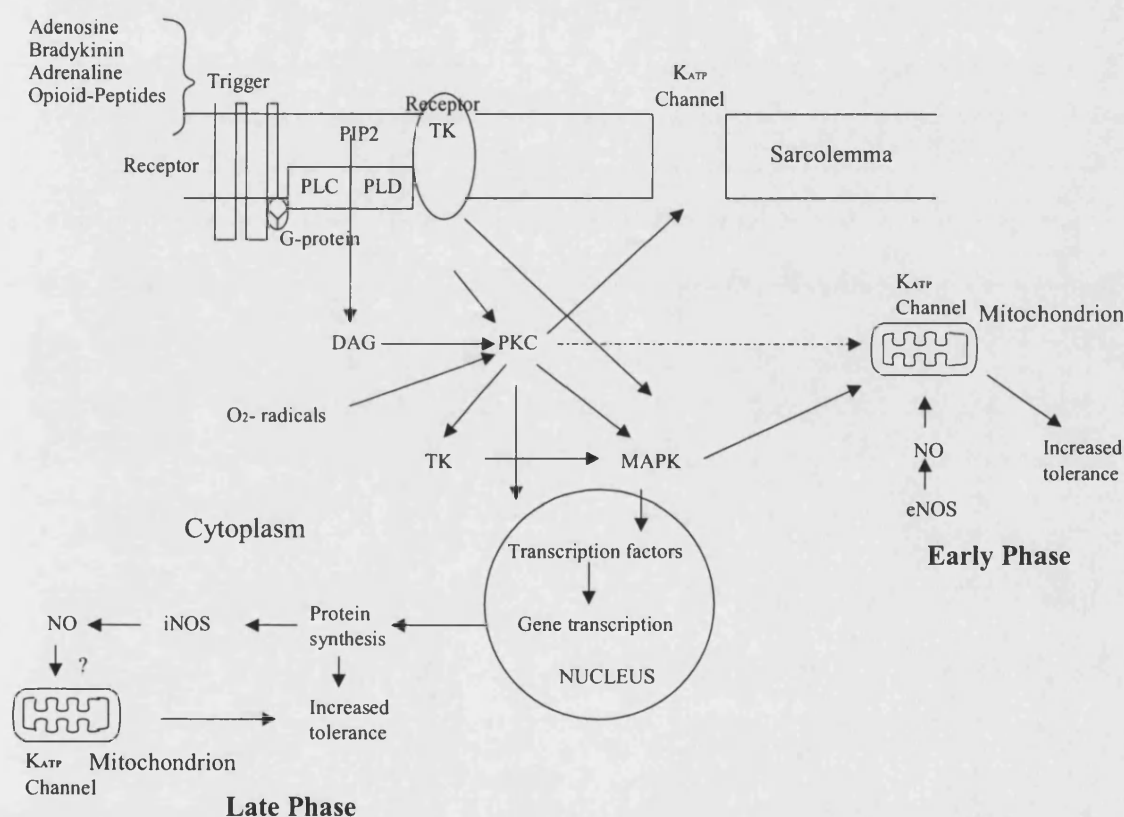


Figure 1-5. Schematic representation of the signalling events that are thought to occur during cardiac preconditioning in the early and delayed phase.

Stimulation of the agonist's receptor triggers, which are coupled to a G-protein, activates either phospholipase C (PLC) or D (PLD). This results in degradation of membrane phospholipids and the formation of diacylglycerol (DAG), the required cofactor for activation (also by reactive oxygen species) and translocation of protein kinase C (PKC) from cytosol to cell membranes. Once activated, PKC can then phosphorylate other end-effectors, possibly K_{ATP} channels via tyrosine kinases or p38 mitogen activated protein kinase to precondition the cell in the early phase with possible

regulation of K_{ATP} channels by NO. The delayed phase occurs with the possible involvement of adenosine receptor activation of PKC, which sets off complex downstream signalling processes. These consist of p38 MAPK, NF- κ B or hypoxia inducible factor-1 resulting in gene transcription, protein synthesis and with the putative regulation of K_{ATP} channel by NO as with the early phase. (Adapted from Yellon and colleagues.)⁸⁶

During the late phase of preconditioning, it is believed that the involvement of the cellular memory results in protein synthesis and therefore takes time to occur hence protection also requires time. It is the production of cytoprotective proteins such as the heat shock family (HSP70, 60, 27, 32 and 90) and antioxidant enzymes such as manganese superoxide dismutase (MnSOD), catalase, glutathione peroxidase/ -reductase and alkyl hydroperoxide reductase that are instrumental in protecting the cell. Some researchers speculate that oxidative stress may trigger the production of these protective proteins.⁹⁸ The enzyme NO synthase appears to be involved⁹⁹ and Guo and colleagues¹⁰⁰ have demonstrated that iNOS is an essential mediator of delayed preconditioning. They disrupted the iNOS gene that abolished the infarct sparing effect of late preconditioning. In rabbits, Xuan and co-workers demonstrated that the biphasic activity of NOS isoforms, eNOS and iNOS served as a trigger and mediator of the late phase of preconditioning respectively.¹⁰¹ Cyclooxygenase-2 (COX-2) induced protection is due to increased production of prostanoids mainly PGI₁ and PGI₂ mediated via δ -opioid receptor induced cardioprotection.¹⁰² Baxter has commented on the fact that even before preconditioning was defined, prostanoids were successfully used for many years to induce delayed preconditioning.⁹⁷ Transient ischaemia-reperfusion that is associated with preconditioning activates a complex cascade of signalling events associated with delayed preconditioning. These include cellular kinases and cardioprotective transcription factors. PKC is known to be involved in the early preconditioning phase,¹⁰³ but it was Baxter and colleagues who demonstrated its important role in delayed preconditioning,⁹⁷ by inhibiting PKC resulting in the abrogation of protection. There are many different isoforms of PKC in differing species but it appears that PKC ϵ is required for delayed pc which is activated by the NO generation.^{103,104}

An end effector of preconditioning has not been clearly identified, although there have been many suggestions of possible candidates, such as the ion channels occurring in the

cell membrane and mitochondria. These are known as the ATP-sensitive potassium (K_{ATP}) channels. Although these channels are known to be cardioprotective in classical preconditioning, there is a lot of speculation as to their role in delayed preconditioning. A lot of attention has been focused on the mitochondrial inner membrane rather than sarcolemma or cell membrane K_{ATP} channels, as K_{ATP} inhibitors for mitochondria and sarcolemma have provided evidence that inhibition of mitochondrial K_{ATP} channel has abolished delayed preconditioning. However, Bernardo and colleagues¹⁰⁵ reported that the delayed preconditioning they observed in the rabbit was due to sarcolemma K_{ATP} , a result differing from the majority.⁹⁷ The actual mechanism of protection has not been elucidated, but as mitochondria can exist in a low conductance state whereby the mitochondrial permeability transition pore (MPTP) can reversibly open and close for short periods without causing mitochondrial swelling or cytochrome c release. This allows mitochondria to purge itself of excess metabolites and ions specifically Ca^{2+} , whilst others state that regulation of K^+ ion influx/efflux by K_{ATP} opening is another possible mechanism which ultimately results in better preservation of ATP energy stores.⁸⁸

Preconditioning in humans has undergone extensive research, but results are hard to define as patients with CD often have collateral circulation that can give false readings. Moreover, using infarct size as a measure of injury is not a practical endpoint. There is evidence of naturally occurring preconditioning in humans. Patients who experience an episode of angina prior to a myocardial infarction (preinfarction angina) often have a better prognosis. In the “warm up angina” phenomenon, patients have painful episodes of angina in the morning but as the day progresses their symptoms improve. There is also evidence that preconditioning can occur during coronary artery bypass surgery and percutaneous transluminal coronary angioplasty (PTCA).^{86,88,97} Pharmacological preconditioning as opposed to using ischaemia (because of the risk of contractile dysfunction or stunning) is more effective, as drugs can activate specific protective pathways, although data from clinical trials has proved to difficult to interpret.^{79 97 88}

1.4.9 Cyclophilin

Some proteins, which contain Xaa—Pro peptide bonds, need *cis-trans* isomerisation in order to achieve their native state.¹⁰⁶ In the fungus *neurospora crassa*, mitochondrial cyclophilin 20 (now more commonly termed cyclophilin-D or cyclophilin-3) binds to mitochondrially-imported polypeptides that require proline isomerisation. These authors determined that cyclophilin 20 catalyses the rate limiting step in this folding pathway since in mutated *neurospora crassa* lacking cyclophilin 20, folding of the imported polypeptide was significantly delayed.⁶⁸

Isomerisation is accomplished by a family of *cis-trans* isomerases called cyclophilins.^{67,107} Other closely related members are FK506 binding proteins (FKBP) and the parvulins. Cyclophilins and FKBP cannot be defined as true molecular chaperones,¹⁰⁷ because they do not increase the yield of a reaction, but only the rate. All peptide bonds in proteins exist in a partial double bond form; existing mostly in the *trans* isomer form. In peptide bonds adjacent proline residues exist in the *cis* state and need to be isomerised in order to achieve their fully functional form. PPIases catalyse the *cis* – *trans* isomerisation of these bonds and accelerate the rate of the reaction.¹⁰⁶ They were identified as the receptors for the immunosuppressant drugs cyclosporin A (CsA) and FK506 respectively. When these immunosuppressants bind to their respective receptors, PPIase activity is inhibited. Hence it has been proposed that PPIase activity must be involved in the immunosuppression mechanism; however, other compounds that inhibit PPIase activity lack immunosuppressant actions^{108,109} so this is in doubt.

The actual mechanisms of immunosuppression are not now thought to be linked to their PPIase enzymatic activity. They probably involve stimulation of the T-cell receptors by

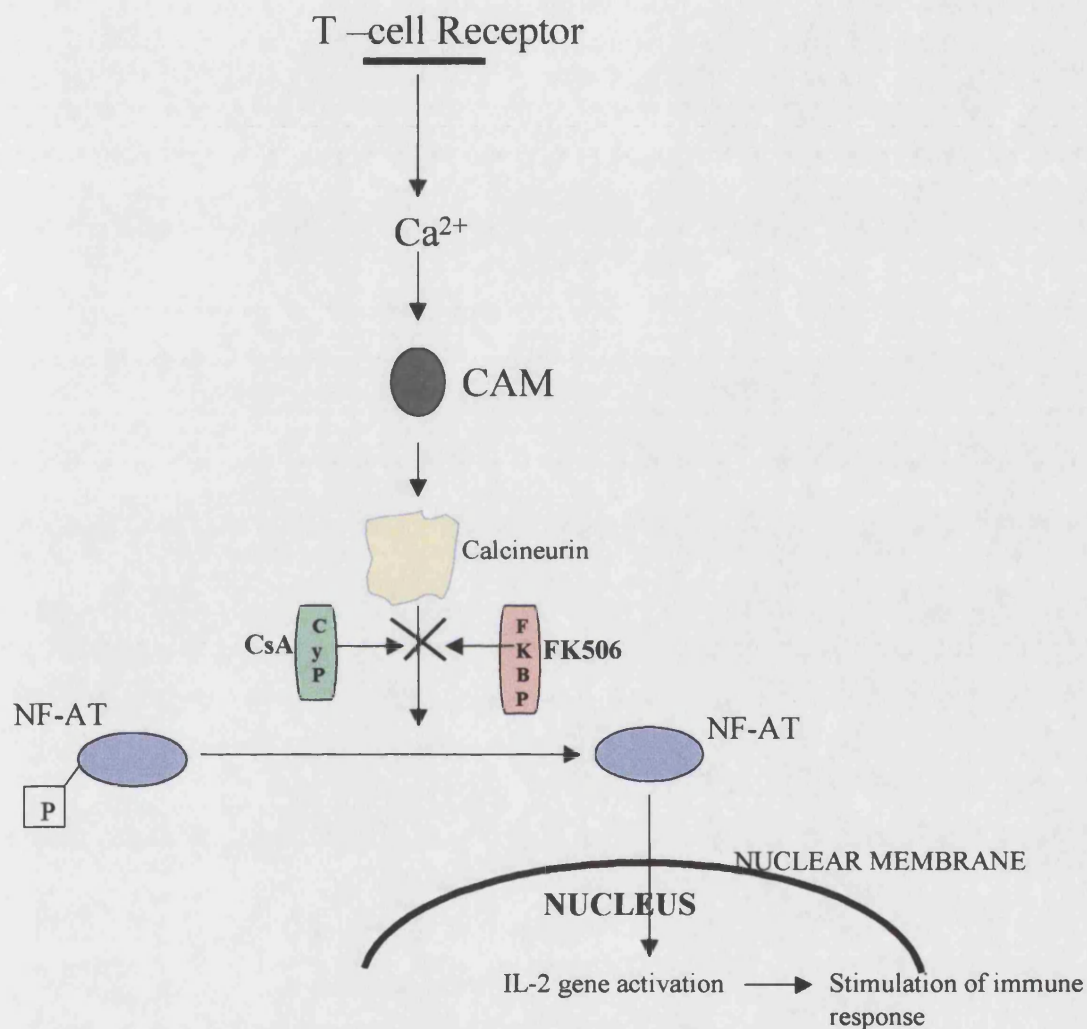


Figure 1-6. Diagrammatic demonstration of T-cell inactivation by CsA

Antigen binding activates the T-cell receptor which causes an increase in Ca^{2+} which acts through calmodulin (CAM) thereby activating Calcineurin resulting in the dephosphorylation of NF-AT. NF-AT must be dephosphorylated before it can enter the nucleus to activate the transcription factor IL-2 resulting in T-cell proliferation. The dephosphorylation of NF-AT can be blocked by the binding of CsA to cyclophilin (CyP) or FK506 to FK506 binding protein (FKBP), forming complexes that prevent this dephosphorylation activity of Calcineurin and subsequent translocation of NF-AT to the nucleus. (Modified from review by Nabel)¹¹⁰

antigens resulting in increased cellular Ca^{2+} levels that then activate a Ca^{2+} dependent protein phosphatase (calcineurin), which (Figure 1-6) in turn dephosphorylates the transcription factor, nuclear factor of activated T-cells (NF-AT).¹¹¹ This in turn allows translocation to the nucleus and activation of the transcription factor interleukin-2 (IL-2) and other cytokine genes resulting in T-cell clonal expansion. The binding of both FK506 and CsA to their respective receptors inhibits activation of calcineurin thus preventing dephosphorylation of NF-AT. IL-2 gene activation is regulated by NF-AT and therefore it is reliant on the translocation of NF-AT to the nucleus to stimulate the immune response. NF-AT must be dephosphorylated for it to enter the nucleus, if this is prevented by calcineurin inhibition, immunosuppression results.¹¹²

Cyclophilins are comprised of: CyP A (18 kDa) which is localised in the cytoplasm; CyP B (21kDa) found in the endoplasmic reticulum, CyP C (21kDa); murine and (CyP-D) CyP 3 (18 kDa) localised to the mitochondrion; and CyP40 which is part of the HSP90 steroid receptor complex.¹¹³ The ubiquity of their expression would suggest that they might have many functions in the cell. Cyclophilins have been reported to possess nuclease activity, which is unrelated to their PPIase activity. Cyclophilins are structurally similar to NUC18 (18 kDa) nuclease isolated from apoptotic rat thymocytes and can degrade DNA.¹¹⁴

Ischaemia/reperfusion injury is characterised by collapse of the mitochondrial membrane potential due to the formation of the mitochondrial permeability transition pore.¹¹⁵ High membrane potential is necessary to maintain ATP synthesis; however due to a dramatic increase in membrane permeability, mitochondrial permeability transition (MPT) occurs, which is the process allowing solutes less than 15 kDa to pass across the membrane. This results from elevated levels of Ca^{2+} and the presence of free radicals in the mitochondria undergoing reperfusion after a long ischaemic period. The formation of the MPTP is often an indicator of the change from reversible to irreversible cell injury.^{116,117} However, despite the role proposed by Halestrap and colleagues whereby CyP-D promotes MPT, overexpression of CyP-D has recently been shown to be protective.^{56,118}

1.4.10 Nitric Oxide

Nitric oxide (NO) is one of the most potent and most recognised endogenous vasodilators in existence.¹¹⁹ NO is an unstable, poisonous free radical gas which is a constituent of the pollutant air¹²⁰ and was originally discovered by Joseph Priestly in the early 1770's.¹²¹ Numerous cell types can produce NO including vascular smooth muscle cells, neutrophils, liver and heart cells.^{122,123} It has a short half-life, reacts with oxygen-derived free radicals, and plays an extensive role in human biology, with outcomes ranging from pathology to homeostasis.

NO is generated from the oxidation of the substrate amino acid L-arginine and an enzyme, NO synthase (NOS) of which there are three isoforms namely neuronal NOS (nNOS) or NOS3, endothelial or constitutive NOS (ecNOS) or NOS1 and inducible NOS (iNOS) or NOS2.¹²⁴ Due to their omnipresent expression, these enzymes have diverse physiological actions. Furchgott and co-workers suggested that endothelium-derived relaxing factor (EDRF) might be NO;¹²⁵ Palmer and colleagues determined that NO in the vascular endothelium was identical to EDRF.¹²⁶

When produced in the presence of ROS species or in excess, NO induces apoptosis and displays neurotoxicity in neuronal cells.¹²⁰ This neurotoxicity is known to be mediated by OONO⁻, formed from NO and O₂⁻.¹²⁷ This discovery led many investigators to believe that NO was responsible for beneficial biological functions whereas peroxynitrite mediated cytotoxic functions especially as it can induce the formation of MPTP. This analysis was later found to be too simplistic.¹²¹ NO inhibits respiration by inhibiting the electron transport chain at complex IV where it competes with O₂ for binding to cytochrome oxidase in the mitochondrion.¹²⁸ As mitochondrial respiration is the main source of energy production in mammals and the fact that mitochondria are involved in apoptotic and necrotic cell death, led to excitement in the scientific community in 1994 when it was discovered that very low levels (nanomolar) of NO was possibly a physiological regulator of mitochondrial function.¹²¹ This study demonstrated that exogenously applied nanomolar concentrations of NO was a reversible and

competitive inhibitor of cytochrome oxidase activity which was later found not to be tissue or species specific. Ever since these discoveries, many researchers have been investigating NO in different organs of the human body. NO has many biological functions, of which some are mediated through the second messenger guanosine 3'5'-cyclic monophosphate (cGMP) by the action of the enzyme guanylyl cyclase.¹²⁸ In the brain, NO plays a role in neurotransmission.¹²⁴ NO has been found to be involved in many diseases such as sickle cell anaemia,¹²⁹ as well as in various neuronal disorder such as Parkinson's disease.^{120,130} Robert Furchgott's discovery in 1980 was recognised in 1998 when he was awarded the Nobel Prize in Medicine. In the same year Pfizer marketed the drug Viagra for erectile dysfunction, which enhances the physiological action of NO by preventing the breakdown of cGMP¹³¹.

There are many reports linking NO in the mechanisms of preconditioning. Over a ten-year period the interest in NO in myocardial ischaemia and preconditioning has been growing rapidly. During ischaemia, iNOS is upregulated and NO production is increased which in turn decreases blood flow. These are methods, which help the myocardium to adapt to ischaemia, and may play a role in the mechanism of hibernation⁷³. There has been substantial evidence presented indicating the necessary role of NO in the late phase of ischaemic preconditioning.^{81,104} NO is thought not to be involved in classical preconditioning.^{40,83} However Nakano and colleagues discovered that administration of the NO donor S-nitroso-N-acetylpenicillamine (SNAP) caused the production of free radicals. This in turn resulted in protection possibly due to activation of protein kinase C (PKC). They could not find any evidence of protection from endogenous sources of NO.⁸³ Boli's group have presented evidence of a biphasic protection mechanism of NO synthase isoforms. In rabbits subjected to brief ischaemic periods resulted in upregulation of cNOS lasting for 30-60 minutes, which then subsequently ceased. No upregulation of iNOS activity was noticed until 24 hours later. At this point no cNOS activity was recorded. The mechanism responsible for the delayed upregulation proved to involve generation of NO, oxidant species and PKC activation which played no part in the observed early cNOS activity.⁸⁴

1.5 Hypotheses and aims

Cyclophilins are stress inducible proteins which have been found in every organism. Due to their *cis-trans* isomerase activity, they are classified as molecular chaperones catalysing rate limiting steps *in vitro* and *in vivo* protein folding. We are particularly interested in the functional role of mitochondrial cyclophilin as they form part of the MPTP which is crucial in all death pathways^{34,85} and because they protect against oxidative stress.⁸⁶ Therefore in this research programme I will endeavour to address the following aims and objectives:

- The functional role of cyclophilin and the mitochondrial chaperone complex during ischaemia /reperfusion injury and its involvement in the phenomenon of ischaemic preconditioning.
- Differences in cyclophilin and the mitochondrial chaperone complex activities in different types of mitochondria.
- To investigate the effect of increased CyP-D expression on cell survival after ischaemia/reperfusion injury.

I will try to prove my hypothesis, which states: Cyclophilin-D is an important component of the MPTP and as such plays an important role in cell death pathways.

CHAPTER 2

2. MATERIALS AND METHODS

2.1 Materials

All cell culture manipulations were conducted in a sterile class II culture hood (Jencons, UK). Tissue culture vessels were obtained from Nunc (Costar, Cambridge, MA). All chemicals unless stated were obtained from Sigma (Poole, UK) and antibodies for Western blotting were obtained from Stress Gen Biotechnologies Corp. (Bioquote Ltd, UK) and Calbiochem-Novabiochem Corporation (San Diego, USA).

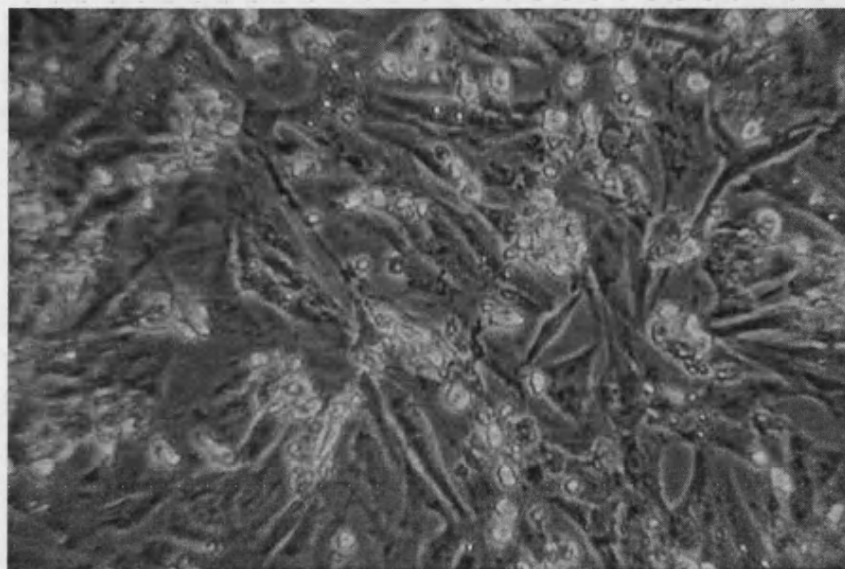
2.1.1 *Preparation of Chemical Reagents.*

The nitric oxide donor S-nitroso-acetyl pencillamine (SNAP) was dissolved in DMSO to make a stock concentration of 1mM, stored at -70°C and then used in the concentrations as described. The cationic flurochrome 3,3'-dihexyloxacarbocyanine iodide (DiOC₆ (3)) was diluted to 40μM in DMSO, which was further diluted to 400nM and used at a final concentration of 0.8 nM in medium. The immunosuppressive cyclic oligopeptide cyclosporin A (CsA) was dissolved in ethanol and added to cell solutions for a final concentration of 30nM.

2.1.2 *Cell Biology*

H9c2 cells were obtained from the American Type Culture Collection (CRL-1446, ATCC Rockville, MD, USA). These myoblasts are large flat spindle - shaped cells with

prominent nuclei that grow in unorganised arrays at low densities, but upon confluency they form organized identical rows. When confluent, they undergo morphological changes becoming net-like in appearance, forming myotubules following fusion providing they are not senescent.



A

Micrograph A. Typical phase contrast of primary rat neonatal cardiomyocytes

This Micrograph is demonstrable of a typical phase contrast image of cardiomyocytes after 24 hours in culture.

They were grown in full growth medium (FGM), Dulbecco's Modified Eagle Medium (DMEM) with sodium pyruvate and 1000mg/l glucose (Gibco-BRL) and 10% (v/v) fetal calf serum in 95% atmosphere and 5% CO₂. These cells were subcultured every 3-4 days and not used beyond passage 29. They were originally derived from a section of a 13-day embryonic rat heart and possess more skeletal than cardiac muscle properties. Due to extensive dedifferentiation they cannot be considered as a true model of mammalian myocardium.¹³² Therefore we also used a primary rat neonatal cardiomyocyte model, which better represents a truly myocardial cell population.

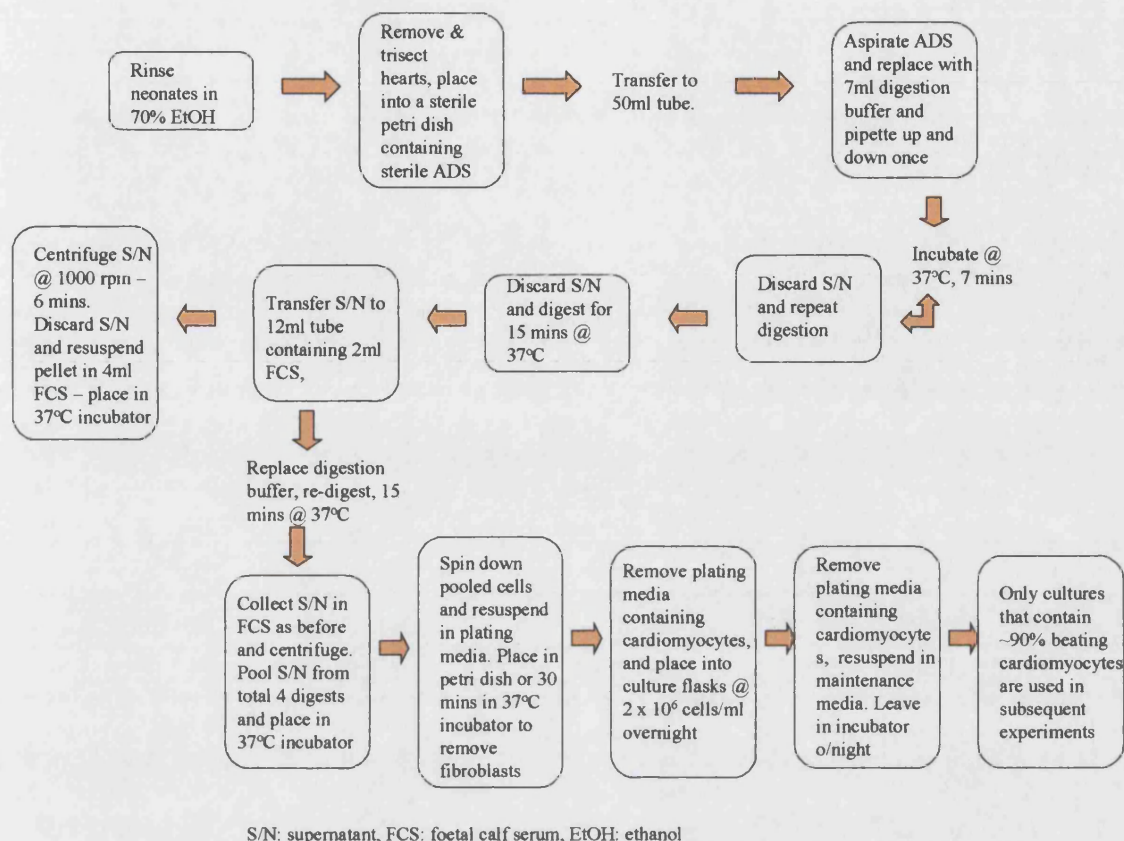


Figure 2-1. Flow chart representing a rat neonatal cardiomyocyte isolation procedure.

Using this rat neonatal cardiomyocyte isolation procedure a total of $48\text{--}72 \times 10^6$ cells can be isolated from 25 – 30 Sprague – Dawley neonate hearts.

These cells were isolated from three litters of between 25 to 30, 1- 3-day-old Sprague – Dawley neonates using collagenase/pancreatin digestions (Figure 2-1). Their hearts were quickly removed, trisected and immersed in ADS, a HEPES buffered salt solution, ⁸⁸ After four digests in 500µl pancreatin plus 30 mg type II collagenase (Worthington Biochemicals, UK) per 100ml ADS, the myocytes were resuspended in plating media 4:1 DMEM/ Medium 199 (Gibco-BRL, UK) with the addition of 1% penicillium/streptomycin, 5 % fetal calf serum, 10% horse serum and preplated into 100mm petri dishes for 30 minutes in order to remove any contaminating fibroblasts.

The densely enriched myocyte population was then seeded into gelatine coated tissue culture plates at a concentration of $1 \times 10^6/\text{ml}$ for 24 hours, whereupon the medium was changed to maintenance medium comprised of virtually serum free DMEM/ Medium 199, thereby reducing fibroblast growth.¹³³ After 24 hours in maintenance medium they were used in experiments only if ~90% were beating (Micrograph A).

CV-1 origin, SV40 (COS) cells (of which there are 3 clones 1, 3 and 7) are immortal fibroblasts originally derived from kidneys of the African green monkey. This expression system is ideal for transient transfections because they are easily transfected. These cells grow quickly to produce substantial quantities of recombinant proteins.

2.1.3 Long-term Storage of Commercial Cell Lines

For long term preservation, cells from a T75 culture flask were trypsinised, pelleted and resuspended in 1ml freezing mixture (8ml foetal bovine serine (FBS), 1ml dimethyl sulfoxide (DMSO) and 1 ml medium) that were transferred to 1.5ml cryotubes. They were then wrapped in tissue paper and kept at -70°C for short-term storage (2 or 3 months). For long-term storage (4 months or more) they were placed into liquid nitrogen tanks (-196°C). When required cells were recovered by warming rapidly (within 1 minute) in a water bath at 37°C , spun and after aspiration of the freezing mixture (to free the solution of DMSO), the cells were resuspended in the appropriate media and seeded into tissue culture flasks.

2.1.4 Rat Left Anterior Descending (LAD) Coronary Artery Occlusion Model

All the rat hearts used were obtained from female adult Sprague-Dawleys of which there were six experimental groups, each rat weighed between 250 to 300g. Myocardial Ischaemia was induced by left anterior descending (LAD) coronary artery occlusion as described in the experimental procedure below.

*2.1.5 Surgical preparation**

Following anaesthesia with hypnorm and diazepam, a surgical incision was made in the breastbone and the chest area was opened. The rib cage was retracted back over the head and the heart was positioned such that the LAD was exposed. A 7/0 silk suture (W817) was tied loosely around the LAD and attached to a snare wire. A snugger tube was pushed over the wire. By pulling the snare wire and at the same time pushing the tubing onto the heart resulted in a gentle occlusion. Placing a small artery forceps around the snugger tube trapped the suture around the LAD thereby securing the occlusion. Reperfusion was performed by removing the forceps and pulling the snugger tubing away from the heart, loosened the suture around the LAD, allowing reperfusion of the ischaemic area. A group of rats underwent the above procedure except for the LAD occlusion and reperfusion stage and thus served as controls.

*All surgical procedures were performed by Milan under a home office licence.

2.1.6 Determination of Area at Risk (AAR)

Finally to determine AAR the femoral vein was cannulated and clamped off. A syringe was then attached containing Evan's blue dye. The LAD was re-occluded and 1ml of Evan's blue dye was infused into the heart. The heart was removed and the AAR was determined by negative staining. The non-blue area (left ventricle) was then removed for mitochondrial isolation.

2.1.7 Experimental Groups

Group1 (Control)

Control rat hearts (n=4) were treated as above but the silk suture was loosely attached around the LAD coronary artery for 60 minutes. The left ventricle was then surgically removed for mitochondrial isolation.

Group 2. 60 minutes LAD occlusion

Rat hearts (n=4) were subjected to 60 minutes LAD occlusion as described (section 2.1.5) and the infarct size and risk area was evaluated via a perfusion technique using Evan's blue dye. The left ventricle was removed for mitochondrial isolation.

Group 3. 60 minutes LAD occlusion and 15 minutes reperfusion.

Rat hearts (n=4) were subjected to 60 minutes LAD occlusion as described (section 2.3.1) with 15 minutes reperfusion. As above Evan's blue dye was used and the left ventricle was removed for mitochondrial isolation.

Group 4. (control)

Rat hearts (n=4) were treated as in Group 1 (control) except silk suture was loosely attached around LAD for 90 minutes. Hearts was then surgically removed for mitochondrial isolation.

Group 5. 90 minutes LAD occlusion

Rat hearts (n=4) were subjected to 90 minutes LAD occlusion and then treated with Evan's blue dye as above and removed for mitochondrial isolation.

Group 5. 90 minutes LAD occlusion with 15 minutes reperfusion

Rat hearts (n=4) were subjected to 90 minutes LAD occlusion and 15 minutes reperfusion followed by treatment with Evan's blue dye as above and the left ventricle was removed for mitochondrial isolation.

2.2 Biochemical assays

2.2.1 Determination of Peptidyl - Prolyl Isomerase (PPI-ase) Activity

The basis of this assay is the measurement of the time course of the rate of conversion of the *cis* to *trans* conformer of the substrate N-Succinyl-Ala-Ala-Pro-Phe p-Nitroanilide (pNA) because the applied isomer specific chymotrypsin will only cleave pNA when it is in the *trans* conformer releasing a chromogenic dye that can be measured spectrometrically. Since the spontaneous *cis-trans* isomerisation of pNA was found to be too fast for reliable calculation of rate constants at room temperature, the assay is now carried out at or below 10°C. The oligopeptide pNA contains a prolyl bond, where the *cis* form is used as substrate in protease-coupled PPIase assays. It consists of a mixture of two conformers, *cis* and *trans*, in solution. In some solvents the percentage of the *cis* conformer is in the range of 5-35% whereas dissolving pNA in Trifluoroethanol (TFE) containing 235- 470mM LiCL, has been found to increase the *cis* conformer to 50% and in some circumstances 70%. ¹³⁴ As detailed below, PPIase activity was measured with bovine α -chymotrypsin as the isomer-specific protease.

Cells were trypsinised and washed in PBS and spun down at 1500 rpm for 5 minutes. They were resuspended in (depending on pellet size) cold ~0.5-2ml 35mM HEPES buffer in 2ml microfuge tubes. They were then freeze- thawed in liquid nitrogen and placed immediately into a 37°C water for duration of 30 seconds each time (repeated 4 times) rupturing the cell membranes. The cells were spun down for 10 minutes at 3000rpm (4°C) and the supernatant was transferred to clean 2ml microfuge tubes. From the lysate the protein concentration was ascertained (Biorad protein assay). Generally 20 μ g cell lysate was used in each assay. HEPES buffer, 100 μ l α -chymotrypsin and cell lysate were added in a cuvette to a volume of 990 μ l. They were cooled to 4°C, 5 minutes before the addition and rapid mixing of 10 μ l of the substrate pNA (at a concentration of 80mM in 470mM LiCl/TFE) to the mixture. This initiated the reaction, which was recorded for two minutes on a spectrophotometer at an absorbance of A₃₉₀ after which the process was repeated twice more. Each sample was assayed three times

in order to determine an average of the PPIase enzymatic activity that was finally calculated by an enzymatic program (GraFit), which calculated the enzymatic activity based on this equation.

$$K = (k_{\text{obs}} - k_0) / k_0$$

Equation 2-1. Determination of PPIase activity.

Where PPIase activity is determined as (K) arbitrary units and k_{obs} as the first order rate constant of the PPIase catalysed reaction and k_0 is the *cis* – *trans* conversion of the uncatalysed reaction. This method was taken from the protocol of Kofron and colleagues⁸⁹ and Kullertz and co-workers.¹³⁵

2.2.2 Isolation of Mitochondria from Cells

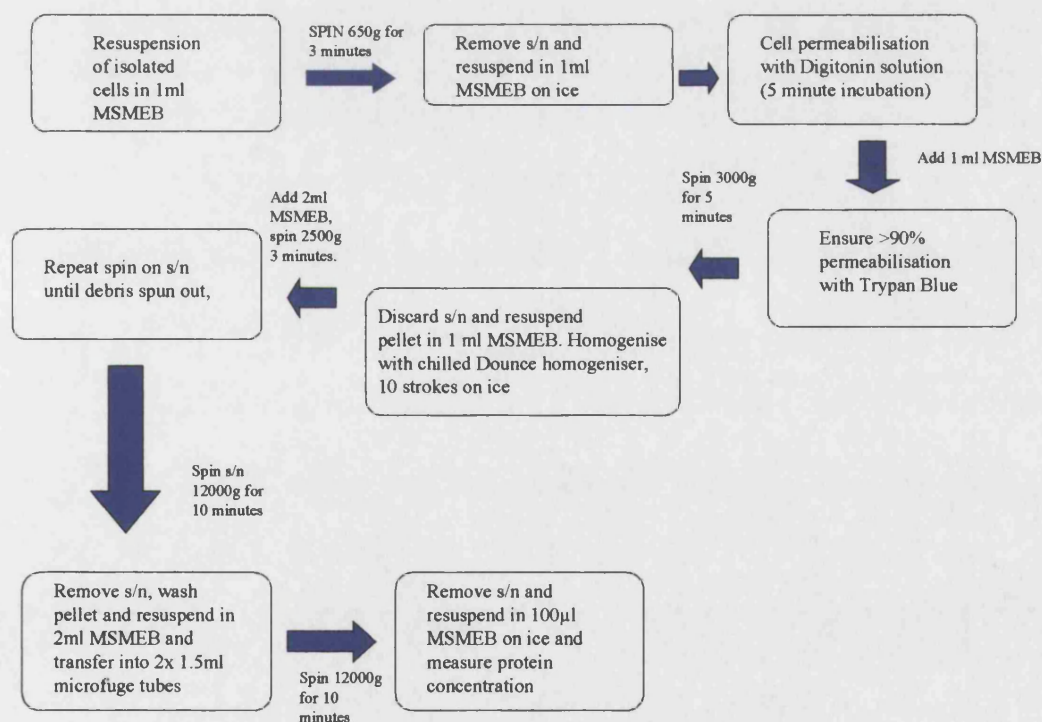


Figure 2-2. Flow diagram of mitochondrial isolation from cells.

Primary neonatal cells were trypsinised and resuspended in MSMEB. After permeabilisation with digitonin, homogenisation and a series of differential centrifugations yielded mitochondria. S/N: supernatant,

Cells were harvested and resuspended in MSMEB (210mM mannitol, 70mM sucrose, 10mM MOPS, 1mM EGTA and 0.5% BSA). The following procedures were all performed at 4°C. Digitonin (5% in DMSO; 0.1mg/ml) was added until >90% cell permeabilisation occurred as confirmed with Trypan Blue exclusion. After the addition of a further 1ml MSMEB, the samples were spun and the supernatant removed. The pellet was homogenised 10 times in a Dounce homogeniser. The supernatant was spun initially at 2500g for 3 minutes and finally for 10 minutes at 12000g. The resulting pellet (mitochondrial fraction) and the supernatant (cytosolic fraction) were washed separately. Protein concentration was calculated using Bio-Rad protein assay (Figure 2-2).

2.2.3 Buffer for Isolation of Subsarcolemmal (SSM) and Interfibrillar Mitochondria (IFM) from Rat Heart Using the Protease Nagarse

Table 2-1. List of ingredients for mitochondrial isolation MSE buffer

MSE Buffer	1 Litre (g)
225 mM Mannitol	40.995
75 mM Sucrose	25.673
10 mM MOPS	2.093
10 mM Tris HCL	1.211
1mM EGTA pH 7.3	.380

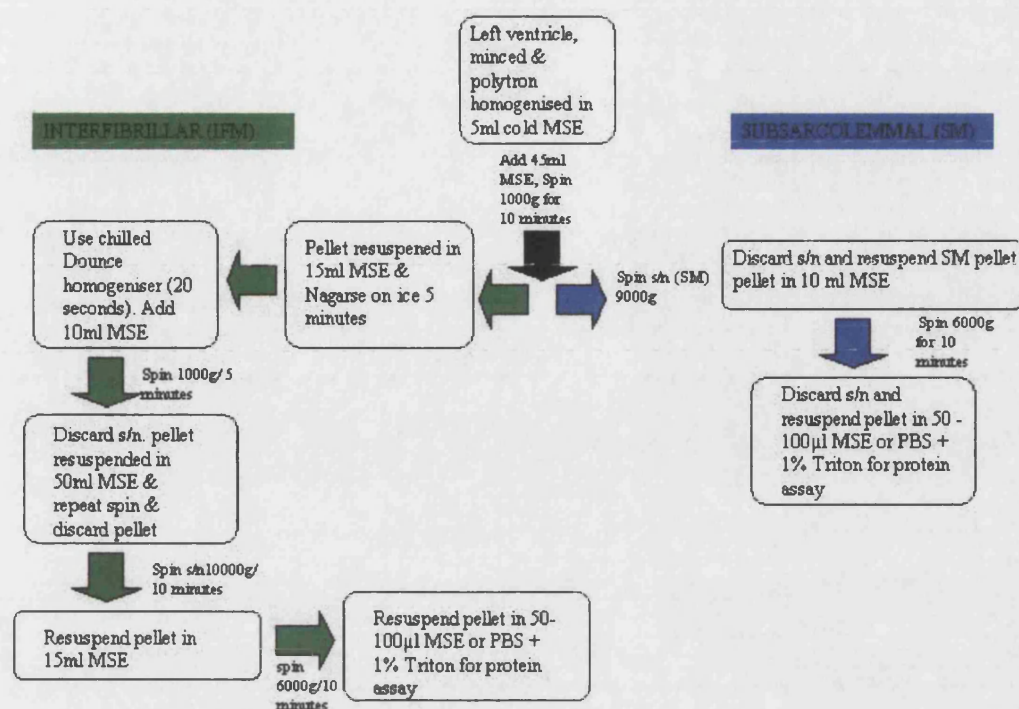


Figure 2-3. Diagrammatic representation of SSM and IFM isolation.

SSM and IFM were isolated from the left ventricle of a female Sprague-Dawley rat. Isolation of SSM was achieved by disruption of the tissue using a polytron tissue processor and differential centrifugations; nagarse digestion of the remaining tissue was necessary to release IFM.

The left ventricle was removed from the hearts and immediately placed into cold MSE buffer (Table 2-1) and washed three times. The ventricles were minced in cold MSE (5ml), homogenised using a polytron tissue processor for 10 seconds and, after the addition of 45ml MSE, were centrifuged at 1000g for 10 minutes. The resulting pellet containing the interfibrillar mitochondria was placed on ice. The supernatant containing the subsarcolemmal (SSM) fraction was centrifuged at 10000g for 10 minutes. The pellet was resuspended in 15 ml of MSE buffer and further centrifuged at 6000g for a further 10 minutes in order to enrich this mitochondrial fraction (Figure 2-3).

In order to release the interfibrillar mitochondria (IFM) from the myofibrils, the 1000g pellet from the first spin was resuspended with 1.5mg nagarse in 5 ml MSE and left on ice for 5 minutes and homogenised for 20 seconds. Then 10 ml MSE was added prior to centrifugation at 1000g. The resultant supernatant was discarded to remove any contaminating SS mitochondria. The pellet was resuspended in 50 ml MSE and re-centrifuged at 1000g for 10 minutes. The pellet was discarded and the supernatant was centrifuged at 10000g for a further 10 minutes and following this the pellet was resuspended in 15ml MSE and centrifuged at 6000g for 10 minutes. These mitochondrial fractions were re-suspended in 50 - 100ul PBS + 1% Triton or HEPES buffer and the protein concentrations were determined.

2.2.4 Cell Viability Assays

Mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2-diphenyl tetrazolium bromide (MTT) formazan assay (Sigma, UK) was one method used to determine cell viability. This was added to the cells at a concentration of 0.5mg/ml and incubated for 30 minutes. After development of the MTT formazan crystals, 0.5ml of stop solution (89ml anhydrous isopropanol; 1% triton; 10ml 0.1N HCL) was added for another 30 minutes until these crystals dissolved. Finally the degree of reduction of the MTT reagent was ascertained spectrophotometrically at 570nm, normalised to control cells in maintenance medium that were considered to be 100% viable.

2.2.5 Trypan Blue Exclusion Assay

Treated cells were incubated with a 0.8% Trypan blue solution and then using a haemocytometer the average number of viable cells able to exclude this dye was calculated and compared to control cells.

2.2.6 *Lactate Dehydrogenase (LDH) Assay*

Cytotoxicity was also determined by LDH (Sigma Diagnostics, UK) release into the medium. This assay measures the quantity of the cytosolic enzyme in extracellular fluids, which is indicative of cell lysis or injury. The reduction of pyruvate due to the oxidation of NADH results in the decrease in absorbance at 340nm and is directly proportional to LDH activity in the medium. In order to measure this cellular activity, medium was collected and LDH activity measured on a plate reader (Molecular Dynamics, UK) and results were calculated as a percentage of the positive control, which was considered to represent 100% LDH release. The positive control were cardiomyocytes subjected to 30 seconds immersion in liquid nitrogen immediately followed by 30 seconds immersion in a 37°C waterbath. Controls were cardiomyocytes incubated in normal maintenance medium for the duration of the experimental periods. This cycle was repeated twice more to invoke cell lysis after which the medium was assayed for LDH activity.

2.2.7 *Flow Cytometry Analysis*

All analysis was performed by fluorescence activated cell sorter (FACS) using a flow cytometer (FACScan) from Becton Dickinson (San Jose, CA) equipped with a single 488-nm argon laser. A minimum of 10,000 cells per sample was analysed and quantified using cellquest software.

Flow cytometry enables the detection of fluorescence and scattered light from the analysed cells by forward-angle light scatter (FSC) and side-angle scatter (SSC). FSC relates to cell size and SSC relates to cell granularity. These parameters are able to distinguish between cell populations and aggregates as well as viable and non-viable cells, as dead cells have a lower FSC signal. This ability is further enhanced by adding propidium iodide (PI) to the sample.

2.2.8 Propidium Iodide Staining

This dye is able to enter dead cells whereupon it binds to nuclear DNA and fluoresces red. Viable cells with intact plasma membranes do not demonstrate any fluorescence.

As a positive control for necrosis, 1×10^6 cells were immersed in liquid nitrogen for 15 minutes and then placed immediately into a 42°C water bath for 15 seconds in order to induce necrosis. This cycle was repeated twice more eventually resulting in >95% PI permeable cells.

Following trypsinisation cells were spun and to the resultant pellet was added $2\mu\text{l}$ of a 2mg/ml propidium iodide solution. These pellets were then resuspended in $500\mu\text{l}$ PBS and analysed by flow cytometry.

2.2.9 Mitochondrial Membrane Potential ($\Delta\psi_m$) Measurements

Formation of the mitochondrial permeability transition pores resulting in a decrease in the mitochondrial membrane potential ($\Delta\psi_m$) was measured using the lipophilic cationic fluorochrome 3,3'-dehexyloxacarbocyanine iodide ($\text{DiOC}_6(3)$),¹³⁶ which accumulates in the mitochondrial matrix of cells depending on their mitochondrial membrane potential. Cells were incubated with 0.8 nm $\text{DiOC}_6(3)$ in tissue culture media for 30 minutes at 37°C . They were then harvested, washed and resuspended in PBS prior to analysis performed on the FACScan flow cytometer (Becton Dickinson, excitation 488nm , emission 495nm)

2.2.10 Simulated Ischaemia Buffer and Hypoxic Chamber

Cells in tissue culture flasks were incubated in a simulated ischaemia (SI) buffer based on a protocol from Esumi and colleagues⁹¹ but modified by Dr. R. Heads.⁸⁸ This consisted of 137mM NaCL, 1.2mM MgCl₂·6H₂O, 2.5mM CaCl₂·2H₂O, 10mM 2-Deoxyglucose, 20mM Na lactate, 12mM KCl, the pH is initially 6.1 and after the addition of 1mM sodium dithionite the pH was adjusted to 6.2 with very dilute NaOH and filter sterilised before being placed onto cells. This in combination with a hypoxic environment was called lethal simulated ischaemia (LSI) was designed to simulate myocardial ischaemic conditions occurring *in vivo*, replicating the approximate concentrations of potassium, hydrogen and lactate ions. Cells were placed in an incubator that had been converted into a hypoxic chamber (Billups-Rothenberg, Del Mar, CA, USA). Over a period of 2 hours the chamber was perfused at a flow rate of 5 litres/hour that was later reduced to 1.5 litres/hour.⁹² The gas mixture consisted of 95% N₂ and 5% CO₂; after the initial two hours the pO₂ in the chamber was reduced from atmospheric pressure 160mmHg to 2mmHg. Whilst the chamber was in use, it was continuously perfused with this gas mixture in order to counteract any O₂ released by the plastic, thereby maintaining the low pO₂. After a defined period, cells were removed from the chamber whereupon they were reperfused with medium for different time points or subjected to further assays.

2.2.11 Measuring Oxygen Levels in the Simulated Ischaemic Buffer

This was performed by making up the simulated ischaemic buffer and immediately after the addition of 1mM sodium dithionite; 1 ml of this solution was transferred into a water-jacketed incubation chamber containing a Clarke electrode (Yellow Springs Instruments Co., Ohio, USA.) The ischaemic buffer was maintained at a temperature of 37°C and the percentage of oxygen in the buffer over a period of 3 - 6 hours was recorded.

2.2.12 Electron Microscopy

Primary neonatal cardiomyocytes growing on 50mm petri dishes after the above treatments were washed with 0.1M-phosphate buffer and then immediately fixed in 3% (v/v) glutaraldehyde (Agar Scientifics Ltd, Stanstead, UK) in 0.1M-phosphate buffer for 2 hours. Following two buffer washes, the secondary fixation process was applied by using 1% (w/v) osmium tetroxide (Agar Scientifics Ltd, UK) with a 1 hour, room temperature incubation. The cardiomyocytes were then washed twice with distilled water and block stained with 4% (w/v) aqueous uranyl acetate (Agar Scientifics Ltd, Stanstead, UK) for 1 hour in the dark, washed in ddH₂O and then transferred into microfuge tubes. These samples were dehydrated through increasing acetone series and infiltrated with 1:1 acetone: araldite CY212 resin (Agar Scientifics Ltd, UK) overnight. Following two changes of fresh resin, each for a minimum of 3 hours, samples were embedded in araldite CY212 resin and blocks were polymerised at 60°C for 18 hours. Ultra-thin (100nm) sections were cut using a diatome diamond knife, on a Reichert-Jung Ultracut E ultramicrotome, floated on to distilled water, collected on formvar-coated copper grids and stained with 2% uranyl acetate for 30 minutes and in lead citrate for 5 minutes. The stained sections were viewed on a Jeol 1200EX electron microscope. (Electron microscopy carried out by Padmini Sarathchandra)

2.3 Molecular Biology

2.3.1 SDS – PAGE and Western blotting

Cells on ice were scraped into cold phosphate buffered saline (PBS), spun down and lysed with PBS + 1% triton. Protein concentration was determined using the Bio-Rad protein assay. The reaction between protein and copper leading to reduction of the Folin reagent is the basis of this method. It has been improved such that the colour development is quicker and more stable when compared to the assay upon which it was founded.

Initially a protein standard curve was obtained using serial dilutions depending on the known concentration of BSA (usually 1mg/ml) diluted to a final volume of 100 μ l with ddH₂O. Then 100 μ l was pipetted into a 96 well plate and the absorbance read on a microplate reader (VersaMax, Molecular Devices, UK) at 570nm. Once the protein curve was constructed, the absorbances of known protein concentrations were compared to those of the unknown samples in order to determine their concentrations. Then equal quantities of protein (usually between 40 μ g - 60 μ g) was combined with Laemmli loading buffer (0.5M Tris-HCL, pH 6.8. glycerol, 10% (w/v) SDS, 2 β -mercaptoethanol, bromophenol blue), boiled for 5 minutes and loaded on 4% stacking gel and separated by electrophoresis on 12 or 15% SDS-polyacrylamide gels (Table 2-2).

Table 2-2. SDS –Acrylamide Gel

List of reagents used to make gels for Western Blot

Reagent	12% Gel	15% Gel	Stacking gel (4%)
ddH ₂ O	3.35ml	2.3ml	6.1ml
1.5M Tris-HCl pH 8.8	2.5ml	2.5ml	-----
0.5M Tris-HCl pH 6.8	-----	-----	2.5ml
10% (w/v) SDS Stock	100 μ l	100 μ l	100 μ l
Acrylamide/Bis (30% stock)	4.0ml	5.0ml	1.33ml
TEMED	5 μ l	4 μ l	10 μ l
10% Ammonium persulfate*	50 μ l	100 μ l	50 μ l

* made fresh daily

The proteins were electroblotted at 30V overnight or 100v for 1 hour onto nitrocellulose membrane (Hybond-ECL, Amersham, UK) and blocked with 5% non-fat milk and 0.05% Tween-20 for 1 hour. The membrane was washed briefly with PBS + 0.05% Tween 20 and 0.1% non-fat milk (PBSTM) on a shaking platform (Janke and Kunkel

GmbH, Germany). The membranes were probed with various antibodies (see Table 2.3).

Table 2-3 List of antibodies used for Western blot

Antibodies	Species	Working dilution	Molecular weight kDA
HSP 10	Rabbit	1:1000	10
*Cyclophilin A	Rabbit	1:1000	18
HSP 60	Mouse	1:1000	60
HSP 70	Mouse	1:1000	70
HSP 75	Mouse	1:1000	75

* It should be noted that where CyP-D protein expression is examined, generally mitochondria has been isolated from the cell, electrophoresed on a SDS-polyacrylamide gel. Following transfer the membrane was probed with Cyclophilin A antibody. As there was no commercially available CyP-D antibody at that time, it was believed that probing mitochondrial protein, where only CyP-D is present with a general cyclophilin antibody would allow CyP-D protein expression to be detected.

Using horseradish peroxidase conjugated secondary antibodies (DAKO); protein bands were visualized by enhanced chemiluminescence (ECL: Amersham, UK).

2.3.2 Plasmid Construction and Transfection -Restriction Enzyme Digest

The CyP-D gene was a gift from Dr D.Bergsma, which was subcloned into pBluescript. This procedure was performed by digesting 5µl (approximately 1µg DNA) of the vector DNA in 1.5ml microfuge tubes (alpha laps, UK) with 2µl buffer E and after the addition of 11µl DEPC H₂O followed by 1µl Eco R1 (12u/µl) restriction enzyme (Promega, UK). The microfuge tube containing the above solutions was incubated at 37°C for 1 hour. To ensure that the cyclophilin inserts had been excised from the vector, 30µl was electrophoresed on a 1% agarose gel.

2.3.3 Agarose Gel Electrophoresis of DNA

Gels were prepared by adding agarose (Sigma, UK), 1% (w/v) to a total volume of 100 ml with 1x TBE buffer and boiled in a microwave cooker for 3 minutes. The agarose solution was allowed to cool to 50°C and then 5µl ethidium bromide (EtBr) was added (0.5mg/ml) prior to casting. Gels were submerged in an electrophoresis tank containing TBE buffer (90 mM Tris; 90 mM boric acid; 2 mM EDTA; pH 8). Loading buffer (1/ 6 volume of 6x stock solution: 0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol FF, 40% w/v sucrose in water) was added to the DNA solutions, which were then transferred into the wells, and electrophoresis was performed at constant voltage of 90v for 1 to 2 hours. The gel was illuminated with short wave ultraviolet light and the DNA bands were visualised by using Polaroid DS 34 camera with a DS H-8 hood (Polaroid UK Ltd., St. Albans, Hertfordshire, U.K.)

2.3.4 Isolation of DNA from Agarose Gel

Low melting point (LMP) preparative grade agarose gels were used for DNA isolation. After the digested insert and pBluescript vector was electrophoresed, the DNA was cut out of the low melting point gel and placed into pre-weighed microfuge tubes. The microfuge tubes were then weighed and the weights of the gel fragments were calculated and the DNA was extracted and purified using the QIA Gel Extraction Kit Protocol (Qiagen, UK). Briefly, 3 volumes of solubilisation and binding buffer QG were added to each volume of the gel (1 volume = 100mg gel). The solution was then heated at 50°C for 10 minutes or until the gel was completely solubilised ensuring that the solution was still yellow confirming that it was at the correct pH. To this, 1 volume of isopropanol was added, mixed and centrifuged for 1 minute.

2.3.5 *Phosphatase Treatment of Vector DNA*

The vector was extracted with an equal volume of phenol/chloroform, spun on a microcentrifuge (Sanyo, MSE, Micro Centaur, UK) at 13000rpm and from the resulting two layers, the aqueous layer was removed and precipitated with 0.1 volume 4M NaCl and 2.5 volumes of absolute ethanol as above. The pellet was then resuspended in 90µl 10mM TrisHCl, pH8, 10µl of 10x phosphatase buffer, 1µl calf intestinal alkaline phosphatase and then incubated at 37°C for 1 hour. The reaction was stopped with the addition of stop solution (1% SDS/10mM EDTA) and 1µl 20mg/ml proteinase K and incubated at 56°C for 1 hour. It was then extracted with an equal volume of phenol/chloroform and precipitated with NaCl and ethanol. The pBluescript vector and insert were then ready to be ligated.

2.3.6 *Ligation of Cyclophilin into pBluescript*

Two microfuge tubes were labelled insert and control, to each was added 1.5µl 5x ligase buffer and 1.5µl DEPC H₂O, 1µl 10mM ATP, 8µl of the vector and into a microfuge tube labelled insert 2µl of the cyclophilin DNA and 2µl DEPC H₂O added to the control microfuge tube instead. Finally 1µl T4 DNA ligase was added to each microfuge tube and they were incubated in a water bath overnight at 15°C. Previously competent cells had been made as described below for the transformation procedure.

2.3.7 *Preparation of Competent Cells*

E-coli DH5α strain were grown overnight in a 2ml culture and 100µl was transferred into 100ml liquid broth (10g tryptone, 5g yeast extract, 10g NaCl and made to 1 litre with ddH₂O, pH 7.5 with 10N NaOH and autoclaved) without antibiotics the following morning. They were grown up until the liquid broth (LB) became cloudy, which on the UV spectrophotometer gave an absorbance of 0.4 - 0.5 % at 550nm. The cells were then spun at 3000rpm for 10 minutes and the supernatant was discarded. The pellet was

resuspended in 4ml 100mM CaCl₂ and stored at -70°C in 200µl aliquots in 0.5ml microfuge tubes until required.

2.3.8 Transformation

The ligation mixes were mixed with 200µl competent cells and immediately incubated on ice for 45 minutes. They were then heat shocked for exactly 2 minutes at 42°C and placed on ice for 5 minutes. Then 800µl LB (no ampicillin) was added to each microfuge tubes and left in a water bath at 37°C for 30 minutes. The cells were resuspended and 100µl was plated onto ampicillin positive agar plates (low concentration) and spread evenly. For pBluescript the LB plates were coated with 50µl of a 0.02% X-gal solution, 4% solution in dimethyl formamide, (20mg/ml) and 50µl IPTG solution (0.1M in ddH₂O, 200mg/ml) 30 minutes prior to use. Cloning into a vector such as pBluescript inactivates the lacZ gene meaning that recombinants were unable to produce β-galactosidase and will not be able to break down x-gal thereby producing white colonies. Non-recombinants, which are able to break down x-gal, then produce blue colonies. The remaining cells were spun down and resuspended in half the original volume and 100µl was pipetted onto agar plates (high concentration) and these were then left upside down in an incubator at 37°C overnight or until colonies grew. Then 10ml LB was inoculated with a single recombinant colony for each sample and left overnight in a shaking incubator (Innova™ 4300, New Brunswick Scientific, UK).

Table 2-4 List of ingredients for reagents for plasmid DNA isolation

Small scale preparation of plasmid DNA (Miniprep)	
Solution 1 (GTE Buffer)	
50mM glucose	
25mM Tris.HCl, pH 8	
10mM EDTA	
Alkaline/SDS solution	
100µl 10N NaOH	
250µl 20% SDS	
To 5ml with ddH ₂ O	
Solution 3	
Glacial acetic acid	11.5ml
ddH ₂ O	28.5ml
	PH 4

All 10 ml cultures were spun down at 3000rpm for 5 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in 100µl cold GTE buffer, vortexed and the contents were then transferred into 2 ml microfuge tubes. To these microfuge tubes were added 200µl alkaline/SDS solution (Table 2-4) and inverting three times mixed them. Then 150µl of cold solution 3 (Table 2-4) was added, vortexed and placed on ice for 5 minutes. The white pellet that forms (cell debris) was spun down and the supernatant removed to a new 2 ml tube and mixed with 1 volume of phenol/chloroform/isoamyl alcohol (49:1:1). They were spun at 12000 rpm for 5 minutes at 4°C and the supernatant was once again transferred to a new 2ml tube whereupon it was precipitated with 600µl isopropanol for 5 minutes. After spinning at 13000rpm for 5 minutes at 4°C, the supernatant was removed to a new tube and washed

with cold 70% ethanol and dried. Once the pellet was dried, depending on the size it was dissolved in 30 - 50µl ddH₂O with 1µl RNase A, measured on the spectrophotometer and then digested with the appropriate buffer and electrophoresed on a 1% agarose gel in order to confirm the presence of the insert in this vector.

Initially CyP-D was cloned into pBluescript, which generated enough restriction sites enabling it to be further subcloned into the expression vector pcDNA3 using the BamHI and HindIII site. After its orientation had been confirmed via restriction mapping, pcDNA3-CyPD was ready to be amplified.

2.3.9 Large Scale Preparation of Plasmid DNA

A Qiagen Plasmid Mega-kit was used, according to the manufacturer's protocol, which is based on the modified alkaline procedure¹³⁷ involving the binding of plasmid DNA to an anion-exchange resin. A single colony was picked from the agar plate (containing the vector and insert), inoculated onto a starter culture of 10ml LB with ampicillin for each sample and was grown overnight at 37°C on a shaking incubator. The following day, this culture was then transferred to a previously autoclaved 2 litre conical flask containing 500ml LB ampicillin and grown for 16 hours at 37°C in a shaking incubator. Then 800µl of each culture was mixed with 200µl glycerol in a 1.5ml microfuge tube. These glycerol stocks were immediately placed into freezing boxes and placed into the -70°C freezer. The rest of the cultures were spun down in a Sorvall GSA centrifuge 6000rpm at 4°C for 15 minutes. The supernatant was discarded and, using a pipette boy with a 10ml pipette, the pellet was completely resuspended in 50ml resuspension buffer P1. Then 50 ml of lysis buffer P2 was added and mixed thoroughly by inverting three times and left at room temperature for 5 minutes. Following this, 50ml of chilled neutralization buffer P3 was added and immediately mixed by inverting three times. The samples were placed on ice for 30 minutes and during this incubation period they were mixed six times. Centrifugation was performed at 11500rpm for 30 minutes at 4°C and the lysate was removed to a new tube and respun. The supernatant containing the plasmid was then respun at 11500 rpm for 15 minutes. The lysate containing the

plasmid was carefully poured into a Qiagen-tip 2500 column ensuring that as little of the sediment as possible was transferred. The cell lysate was then filtered through this anion-exchange resin column, which had been pre-equilibrated with 35ml buffer QBT, by gravity flow. Under these conditions the plasmid DNA binds to the anion-exchange resin. In order to wash the resin; 200ml of medium salt buffer QC was poured onto it. This removed RNA, proteins and low molecular weight impurities. Then 35ml of high salt buffer QF was used to elute the plasmid DNA into centrifuge tubes and then desalted by precipitation with 24.5 ml isopropanol at room temperature. After the DNA was pelleted by centrifugation at 11000rpm for 15 minutes at 4°C, the supernatant was discarded and the pellet was washed with 70% (v/v) ethanol at room temperature and spun at 11000 rpm for 10 minutes at 4°C. Following the spin, the resultant DNA pellet was air-dried and dissolved in ddH₂O. After the concentration was measured by UV spectrophotometry, it was digested with BamHI and Hind III restriction enzymes and electrophoresed on a 1% agarose gel to confirm that the insert was present. The purified plasmid was then further purified on a caesium chloride (CsCl) gradient where appropriate.

2.3.10 CsCl Density Gradient Centrifugation

To 1ml of the plasmid prep, 1g CsCl and 120µl EtBr (10mg/ml) was added. The tubes were spun at 5000rpm in order to collect the precipitate that forms as debris on the cell surface. The clear pink solution was transferred to a 2 inch Beckman tube (Beckman, USA). The tubes were balanced to 0.1g of each other and then spun (Ultracentrifuge, TL-100, Beckman, USA) at 15°C for 16 hours at 100,000g. The tubes were removed from the ultracentrifuge and two EtBr bands were visualized. The lower band contains the denser supercoiled plasmid DNA. This band was carefully removed into another tube and mixed thoroughly with an equal amount of water-saturated butanol and spun at 12000rpm for 2 minutes at room temperature in order to remove the EtBr. This was repeated and each time the upper pink organic layer was discarded, until this layer was eventually colourless. The CsCl was then removed by diluting the sample with 2 volumes of water and then 6 volumes of cold ethanol, mixed, incubated for 30 minutes

at 4°C, spun, washed with 70% (v/v) ethanol and then the pellet was dried and resuspended in ddH₂O + RNase A. The DNA concentration was measured on the spectrophotometer and electrophoresed on an agarose 1% gel and then digested with the appropriate restriction enzyme.

It was further subcloned into the expression vector pcDNA3 in the correct orientation for expression into the BamHI and HindIII site. CyP-D was transfected into 3-day-old isolated primary neonatal cardiomyocytes and H9c2 cells using a novel transfection method, which is based on a peptide that complexes well with DNA and possess an integrin targeting domain. This mediates the internalisation of the vector into mammalian cells by integrin cell surface receptors by a phagocytic mechanism.¹³⁸

2.3.11 Transfection of pcDNA3-CyPD in Cardiomyocytes

In an autoclaved 1.5ml microfuge tube, 80µl (40µl/µgDNA) of the integrin targeting peptide was pipetted followed by 1.5µl lipofectin[®] reagent (GibcoBrl, Life Technologies, UK). Then the DNA to be transfected was diluted to 1mg/ml and 2ul was mixed with 200µl optimem[®] to achieve a concentration of 1µg/100µl in a separate microfuge tube. The DNA/optimem[®] and peptide/lipofectin mixes were combined, mixed and left for an hour in order for the peptide to form a complex with the DNA. In order to transfect into 6 well culture vessels, the contents of a 1.5ml microfuge tube was sufficient to transfect into 1 well of a 6 well culture vessel. After an hour, the cells to be transfected were washed with serum free DMEM. Then the contents of each microfuge tube were diluted to 800µl with Optimen[®] and then each well was incubated with the contents of these microfuge tubes overnight at 37°C in incubator. Control cells were incubated with the same quantities of peptide and lipofectin plus empty vector (pcDNA3). The following day the transfection solution was replaced with normal maintenance medium and the cells were left for 24 - 48 hours in order to allow development of protein expression. As a measure of transfection efficiency, green fluorescent protein (GFP) was also transfected which was visualized on a fluorescent microscope (Carl Zeiss, West Germany).

A further construct was made by subcloning the CyP-D insert directly into a vector which contained pIRES -GFP thereby allowing a more direct measure of transfection efficiency. This vector was called pcDCAG-CyP3.

2.4 Statistical Analysis

Data was expressed as mean \pm SEM for at least 2 separate experiments employing cells from different isolations, which were performed by student's two-tailed t-test. When data was expressed by 3 or more separate experiments, any differences between groups were analysed by analysis of variance using the Bonferroni test to account for multiple comparisons. All statistical analyses were performed with a statistical package associated with the scientific graph program Graphpad Prism® version 3 (GraphPad software, San Diego, CA, USA). $P < 0.05$ was considered to indicate a significant difference in mean values.

CHAPTER 3

3. CHARACTERISATION OF AN *IN VITRO* MODEL OF LETHAL SIMULATED ISCHAEMIA/ REPERFUSION INJURY IN ISOLATED CARDIOMYOCYTES

3.1 Introduction

In the heart, loss of oxygen caused by a coronary obstruction reducing the blood flow is termed cardiac ischaemia. Cells will eventually die of oxygen deprivation if the blood flow is not restored. When the blood flow is re-established more cells may die because metabolites and ions produced during ischaemia react with oxygen to produce cytotoxic agents.¹³⁹

Mitochondria are major targets affected by oxidative deprivation. In these ischaemic surroundings, as the oxygen level decreases, mitochondrial oxidative phosphorylation is inhibited. However, anaerobic glycolysis is able to produce adenosine triphosphate (ATP).¹⁴⁰ This pathway also produces lactate, which causes a reduction in intracellular pH resulting in acidosis that eventually inhibits glycolysis and mitochondrial ATP production.¹⁰⁹ Because the demand of the myocytes for energy far exceeds the supply from anaerobic glycolysis, ATP decrease and ADP begins to accumulate in the latter part of the reversible phase of ischaemia, 75% to 80% of the ATP present at the onset of ischaemia has been consumed.¹⁴¹ Lactate accumulation and glycogen depletion increase as a function of the severity of ischaemia. The decreasing ATP concentration inhibits Na^+/K^+ ATPase resulting in a progressive increase of the concentration of cytosolic Na^+ and a concomitant increase of extracellular K^+ . Na^+ , in turn activates $\text{Na}^+/\text{Ca}^{2+}$ and Na^+/H^+ exchange inducing an accumulation of Ca^{2+} and protons. The intracellular Na^+ rise results in a depolarisation of the cell membrane with transient opening of the voltage-dependent Ca^{2+} channel increasing Ca^{2+} flux. The increase in

intracellular Ca^{2+} levels activates the release of various degradative enzymes and leads to the collapse of the mitochondrial membrane potential ($\Delta\psi_m$) in conjunction with a decline in ATP levels.^{25,48,142}

The formation of the mitochondrial permeability transition pore (MPTP) precedes collapse of the $\Delta\psi_m$. The process in which the MPTP forms resulting in massive swelling and depolarisation of the mitochondria commonly referred to as mitochondrial permeability transition (MPT).¹⁴³ Complete hydrolysis of ATP produced by glycolysis as well as uncoupling of any residual oxidative phosphorylation, eventually results in cellular death. Hunter and Howard discovered the MPTP in the 1970's.⁴⁰ At present it has been speculated by numerous investigators that MPTP dysfunction is a determinant in the event of cellular death.^{37,142,144} Since then, the formation of the MPTP has been shown to be inhibited by cyclosporin A (CsA), which binds to cyclophilin D (CyP-D). In binding to CyP-D, CsA inhibits the PPIase activity of CyP-D and also prevents it binding to the adenine nucleotide translocase (ANT). Thus in this manner as already stated, the formation of the MPTP is inhibited.¹⁴² CyP-D, a *cis-trans* isomerase, catalyses the interconversion between *cis* and *trans* conformations of peptide bonds adjacent to proline residues.¹⁰⁶ This protein is an integral member of the pore and therefore affords protection against IR injury when bound to CsA.¹⁴²

A hypoxic chamber was used in combination with a simulated ischaemia buffer^{133,145,146} to simulate the extracellular environment of myocardial ischaemia. This was the ischaemic model developed specially for this study (by our group) in rat cardiac myocytes to investigate $\Delta\psi_m$ and other intracellular changes occurring during myocardial IR injury. Oxidative stress is an essential factor in IR injury causing recruitment of CyP-D to the ANT-VDAC complex,⁴⁴ Increased oxidative stress results in increased binding of CyP-D to this complex.^{54,55}

3.2 Aims

The aims of this study were two fold:

- 1) To determine whether binding and increased levels in CyP-D might be detected as a change in protein expression or PPIase activity.
- 2) To determine whether apoptosis or necrotic cell death predominates in the lethal simulated ischaemia/reperfusion (LSI/R) model

The hypotheses to be tested are that:

- CyP-D binding to the ANT increases with increasing oxidative stress and the integral PPIase activity induces a conformational change to form the MPTP, which is represented by an increase in PPIase activity.
- As the duration of ischaemia increases, cells will die by necrosis rather than apoptosis

3.3 Methods

Primary neonatal cardiomyocytes were isolated as described in Chapter 2. These cells were plated in 6 or 12 well dishes and simulated ischaemia (SI) buffer was added. This buffer mimics ischaemic conditions such as inhibition of oxidative phosphorylation, acidic conditions and lack of oxygen. The ischaemic buffer was prepared from constituents of Tables 3-1 and 3-2 as follows:

The stock basal modified Krebs-Henseleit Buffer (KHB) was made to 10 times the normal concentration (pH 7.5) (Table 3-1). 5ml of this solution with the addition of components from Table 3-2 was diluted with ddH₂O into a final volume of 50 ml.

Table 3-1. List of components used for simulated ischaemia buffer

KREBS-HENSELEIT BUFFER	
137mM NaCl	16g
3.58mM KCl	0.54g
0.49mM MgCl ₂ .2H ₂ O	0.2g
1.8mM CaCl ₂ .2H ₂ O	0.528g
4mM HEPES	1.906g

Table 3-2. List of components used for simulated ischaemic buffer

Reagent List	Quantity Used
ddH ₂ O	45ml
2-deoxyglucose (2-DOG)	82.1mg
Na lactate (30%: 1:2 dilution of sigma 60% stock)	172μl
12mM KCl (100x)	500μl

After the solution was warmed to 37°C, 1mM sodium dithionite was added, and then adjusted to pH 6.3 and filter sterilized. Prior to addition of SI buffer, the cells were washed with PBS; 1ml of SI buffer was added to each well containing neonatal cardiomyocytes and immediately placed into the hypoxic chamber. Sodium dithionite was used as an oxygen scavenger. To determine how effective this oxygen scavenger was at maintaining a hypoxic environment, a Clark type oxygen electrode was used to measure oxygen levels in this buffer over a period of 3 hr.

Table 3-3. Incubation time points for electron microscopy samples

Duration of Lethal Simulated Ischaemia only	Hours of reperfusion
1 hour	-
1 hour	+ 24hr
3 hours	-
3 hours	+2hr*
3 hours	+24hr
6 hours	-
6 hours	+ 24hr

*Only used for the cell viability assays

Primary neonatal cardiomyocytes were incubated for periods of 1,3 and 6hr simulated ischaemia (SI) in the hypoxic chamber (LSI) with reperfusion for 2hr and 24hr reperfusion (LSI/R) as demonstrated in Table 3-3. The floating cells in the supernatant were collected and spun down. The remaining adherent cells were trypsinised and both sets of cells were fixed for analysis by electron microscopy as previously described in Chapter 2.

In proceeding experiments, 1,3 and 6 hour incubations with subsequent reperfusion were used, and from the supernatant and cells collected, the following assays were conducted (Table 3-4).

Table 3-4. List of experimental assays used.

Incubation time periods for LSI and LSI/R assays used for lethal simulated ischaemia/reperfusion experiments

LSI incubation	Duration of reperfusion	LDH	MTT	H ₂ DCFDA	$\Delta\psi_m$
3hr	-	+	+	+	+
3hr	15 min	+	+	+	-
3hr	2hr	+	+	+	+
3hr	24hr	+	+	+	+

The supernatant was collected immediately after LSI and various reperfusion (LSI/R) time points in order to measure viability via the LDH assay. The intracellular HSP70 expression by Western blotting was analysed in the collected samples.

Mitochondrial membrane potential ($\Delta\psi_m$) was measured with DiOC₆ (3) over the same period of ischaemia and reperfusion time points, and mitochondria were extracted in order to assay for PPIase activity. These procedures were carried out as previously described in Chapter 2.

3.4 Results

Reduced oxygen levels in tissues are termed hypoxia and results in numerous pathophysiological conditions such as ischaemia, cancer and atherosclerosis. Incubating isolated cardiomyocytes in hypoxic or ischaemic conditions result in the inducement of genes for HSP's and antioxidant enzymes.¹⁴⁷ In establishing this model cardiomyocytes were initially incubated for various periods in maintenance medium in a hypoxic chamber (described in Chapter 2).

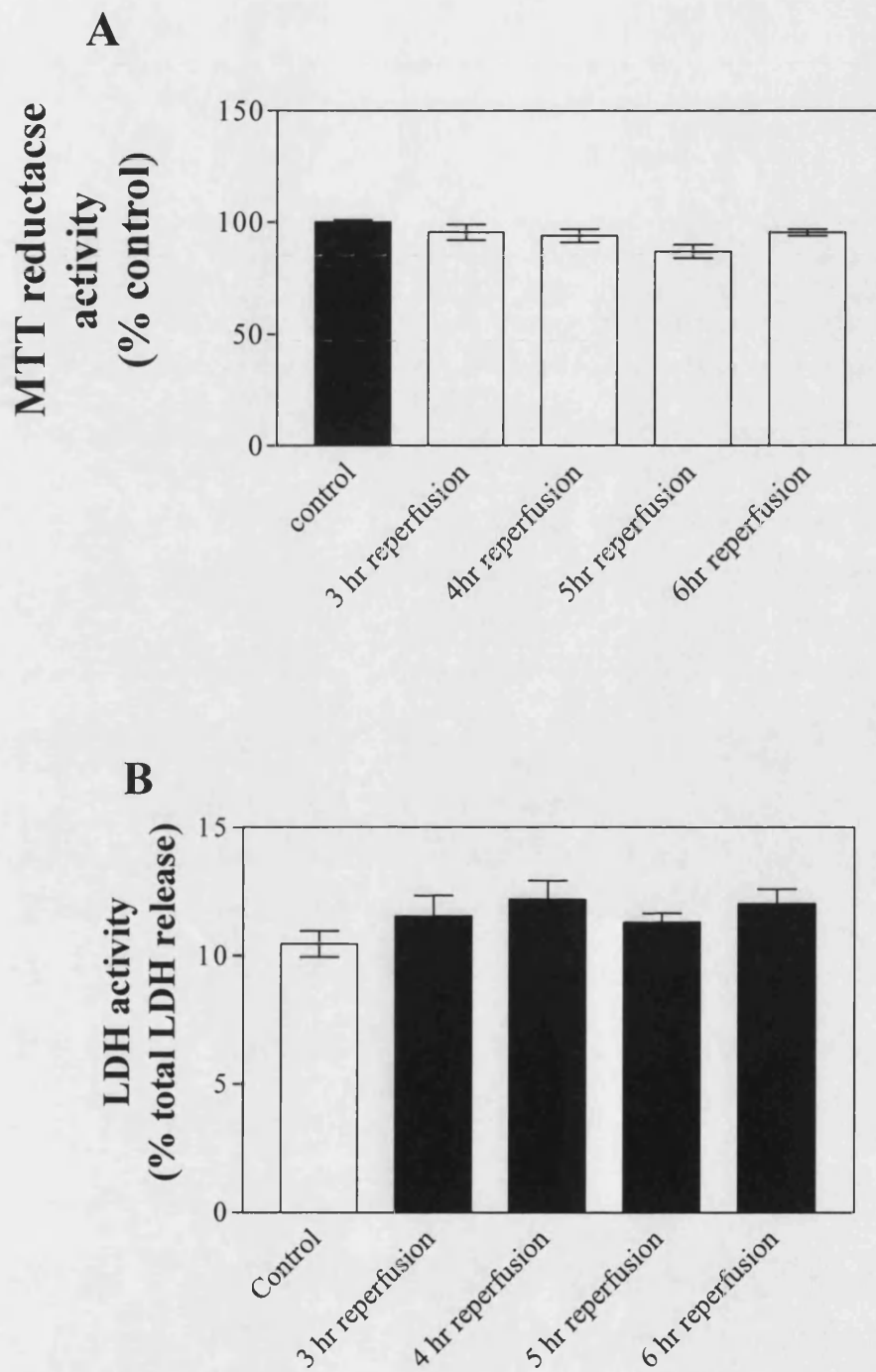


Figure 3-1. Effect of 18 hours hypoxia and differing periods of reperfusion on primary neonatal cardiomyocytes.

Isolated neonatal cardiomyocytes were exposed to 18 hours hypoxia whilst in maintenance medium. The medium was replaced with fresh maintenance medium

(reperfusion) and incubated for 3, 4, 5 and 6 hours. Then the 3-(4,5-dimethylthiazol-2-yl)-2-diphenyl tetrazolium bromide (MTT) formazan assay was performed **(A)** (as described in section 2.2.3) to assess viability. Then the Lactate dehydrogenase LDH assay was performed to assess viability **(B)** (as described in section 2.2.5). Viability of primary cardiomyocytes was not affected by 18 hours hypoxia and up to 6 hours reperfusion. Data are representative of the mean \pm S.E.M. of 4 individual experiments.

Figures 3-1A and 3-1B show that exposure of cardiomyocytes to 18 hours hypoxia and up to 6 hours reperfusion has no effect on cell viability as measured by MTT and LDH assays. These experiments were based on previous work carried out in our Laboratory in which H9c2 cell viability was reduced by 18 hours hypoxia followed by 5 hours reperfusion.¹⁰⁷ We were hoping to perform the same experiments in order to develop a model using rat neonatal cardiomyocytes. However, the above experiments in neonatal cardiomyocytes had no effect when incubated in maintenance medium. An ischaemic buffer was obtained (described in Chapter 2) from Dr Richard Heads (Rayne Institute) which when used in combination with the hypoxic chamber resulted in a decrease in viability of the cardiomyocytes as visualised in Figure 3-2.

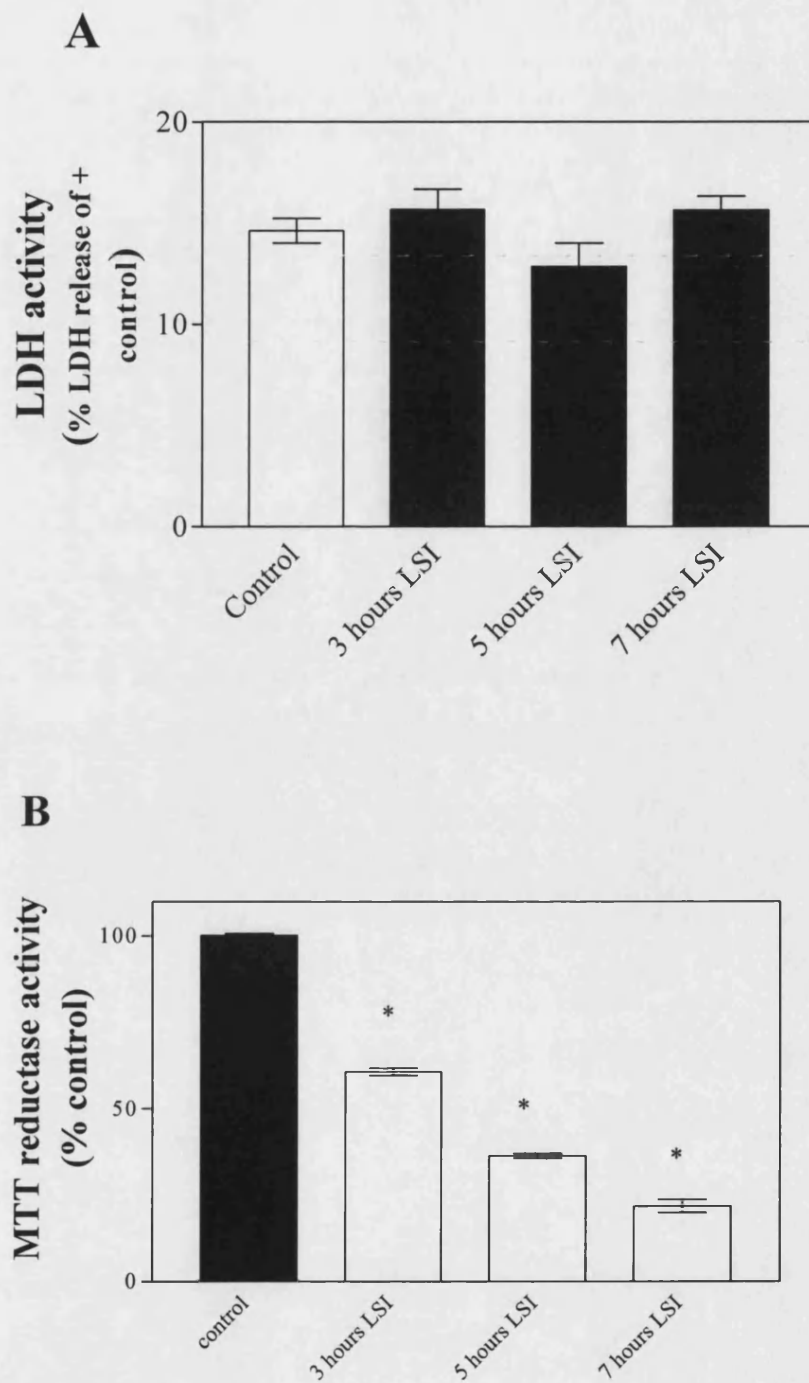


Figure 3-2. Primary rat neonatal cardiomyocytes cells subjected to differing periods of LSI.

An ischaemic buffer in combination with the hypoxic chamber (LSI) reduced the viability of cardiomyocytes in a time dependent manner as measured by the **(B)** MTT assay and as indicated above. **(A)** LDH assay was performed on rat neonatal

cardiomyocytes that had been subjected to LSI for 3,5 and 7 hours. LDH (A) release between the LSI samples and their controls did not demonstrate any significant differences. (B) On the contrary MTT activity between the LSI samples and their controls are statistically different in comparison to its control. The (A) LDH release from the treated samples is calculated as a percentage of LDH of the positive control as demonstrated previously (Figure 3-1B). This result is in contrast to the previous experiments (Figure 3-1A and Figure 3-1B) where the use of maintenance medium had no effect on the viability of cardiomyocytes. Data are represented by mean S.E.M \pm of 6 individual experiments. *P<0.001 vs control

The results from the MTT assay in Figure 3-2B shows the reduction in metabolic activity or viability that decreases with increasing duration of LSI. Interestingly in the corresponding LDH assay (Figure 3-2A) cardiomyocytes appear to be significantly unaffected by this buffer in respect to the control.

Interestingly the following LDH activity graphs (Figures 3-3A and 3-3B) demonstrate statistically significant LDH release in comparison to their respective controls upon reperfusion.

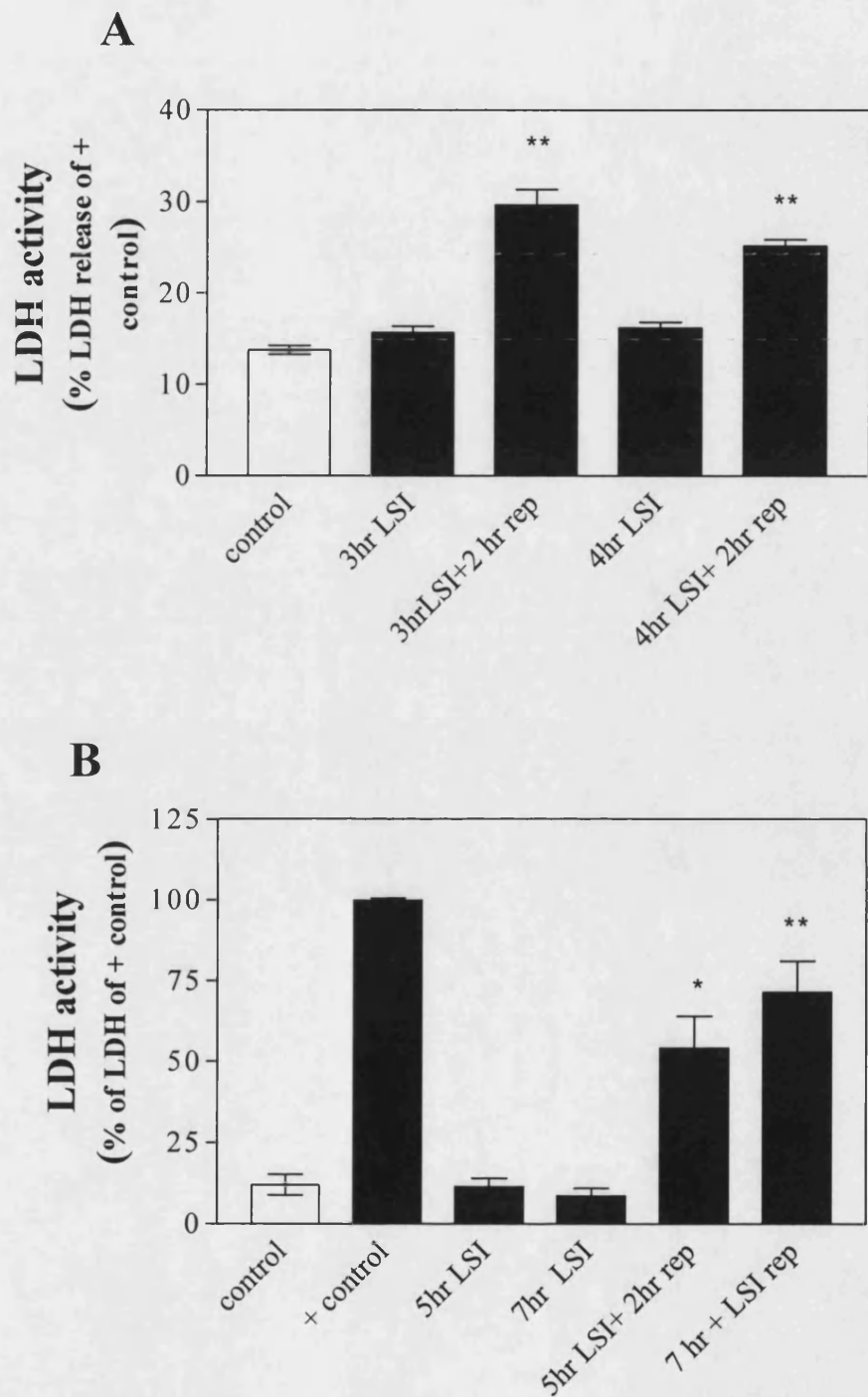


Figure 3-3. LDH activity of cardiomyocytes subjected to LSI/R

Primary cardiomyocytes were subjected to (A) 3 and 4 hours LSI and both were reperfused for 2 hours (LSI/R) in normal maintenance medium. During 3 hours LSI there is no apparent difference between LDH release in comparison to the control, but

upon reperfusion LDH activity is statistically significant after 3 and 4 hour LSI/R. As in (Figure 3-2A), the samples are calculated as a percentage of the positive control. (B) Cardiomyocytes that have been reperfused demonstrate a statistically significant difference in LDH release as opposed to control cells. LSI only treated cardiomyocytes show comparatively the same LDH release as control cells Results are representative of the mean S.E.M \pm of 6 individual experiments. *P<0.01; **P<0.001 vs control

In order to establish the model, it was necessary to expose cardiomyocytes to hypoxia or simulated ischaemia such that a reduction of cell viability of $\sim 50\%$ was attained. This model could then be used to evaluate the effectiveness of preconditioning protocols as well as studying cell death pathways in an *in vitro* cell system, which mimicked myocardial IR. From the combined data from Figure 3-2B and the subsequent LDH assays (Figure 3-3) an initial ischaemic or LSI period of 3 hours was chosen. However, during this time course the hypoxic chamber achieves a hypoxic environment within 2 hours. Figure 3-4 demonstrates that the combination of the oxygen scavenger sodium dithionite and the chamber ensure that hypoxic conditions are maintained throughout the 3 hour time course.

A Clark type oxygen electrode was used to measure oxygen levels in this buffer over a period of 3 hr in order to access how effective the sodium dithionite was at maintaining a hypoxic environment (Figure 3-4).

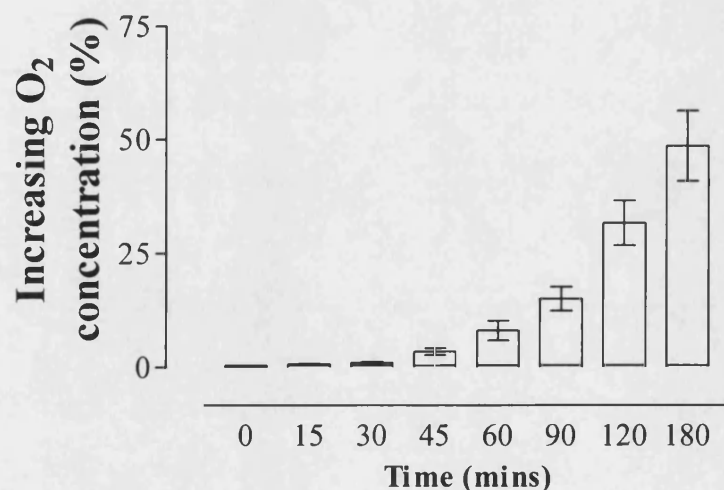


Figure 3-4. Oxygen levels in the simulated ischaemia buffer

The level of oxygen in the simulated ischaemia buffer was measured

against time using a Clark type oxygen electrode after the addition of sodium dithionite. This experiment was conducted in order to ascertain how effective sodium dithionite was at scavenging O₂ in this solution. After 60 minutes, O₂ concentration was at 10%, 30% at 120 minutes rising to 50% at 180 minutes. Data are representative of the mean S.E.M \pm of 4 sets of individual experiments.

This experiment proved that the sodium dithionite was only effective in suppressing O₂ levels for 90 minutes (Figure 3-4). It was necessary to find a way to maintain a low O₂; therefore we used a hypoxic chamber. This device has been proven to lower pO₂ from atmospheric pressure to 2mmHg (as previously explained in Chapter 2) at 37⁰C within 2 hours. However, hypoxic conditions are achieved within 2 hours in this chamber, therefore the use of sodium dithionite will ensure that hypoxic conditions are maintained until the pO₂ levels in the chamber drop to 2mmHg.

3.4.1 Effect of LSI/R on Cell Viability

Primary cardiomyocytes were subjected to 3 hours LSI/R and the viability was assessed with the LDH assay (Figure 3-5). Upon addition of the ischaemic buffer there was an almost immediate cessation in cardiomyocyte contraction or beating; the cardiomyocytes resumed contracting as soon as they were reperfused after 3 hours LSI.

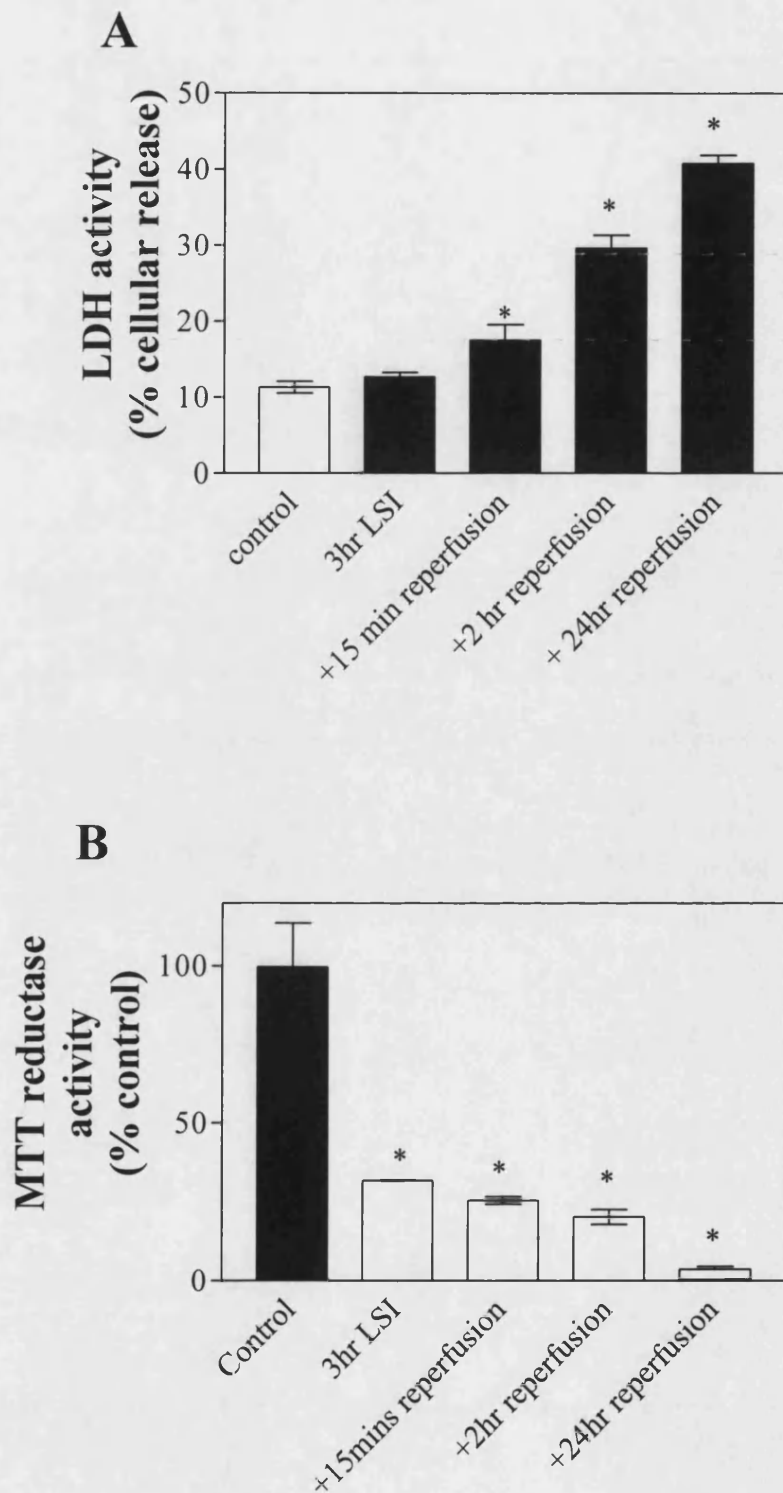


Figure 3-5. Graph depicting viability of neonatal cardiomyocytes.

Survival of cardiomyocytes after 3 hours in LSI and different periods of reperfusion with fresh medium as measured by the (A) LDH assay. As demonstrated in **Figure 3-2A**, these samples are compared to the positive control, which is representative of 100%

LDH release. **(B)** Survival of primary cardiomyocytes that have all been subjected to 3 hours LSI and some have been reperfused (LSI/R) for differing periods as measured by the MTT metabolic assay. This graph shows that 3 hours LSI and 3 hours LSI/R time points are all statistically significant in comparison to the control. Data from each bar are representative of means \pm of 4 independent experiments where significance ‡ represents $P < 0.05$ versus control; * is $P < 0.001$ versus control.

The effect of 3 hours hypoxia with different periods of reperfusion on cell viability is demonstrated above (see Figure 3-5A and 3-5B). After the ischaemic period a time dependant decrease in cell viability was observed as shown in the MTT and LDH cell viability graphs. This demonstrated that 3 hours LSI and up to 24 hours reperfusion was sufficient in this myocardial IR model to allow an adequate reduction in cell viability to be used as a lethal insult in order to determine the effectiveness of preconditioning and other protection protocols. As well as enabling the study of cell death pathways in myocardial IR.

In the LDH assay 3 hours ischaemia and 0 hour reperfusion exhibited no change in release of the enzyme LDH (Figure 3-5A). In comparison we see a dramatic drop in the degree of viability as observed with the MTT assay after 3 hr ischaemia only. As MTT assay is dependent on mitochondrial reductase activity¹⁴⁸ and during ischaemia there is a reduction in oxygen and ATP, metabolic activity decreases which is visualised as a cessation in contractile activity. The LDH assay measures the reduction of pyruvate to lactate catalysed by LDH in the supernatant resulting in the oxidation of NADH to NAD that can be measured at 340nm. In other words it measures the release of lactate dehydrogenase from cells with compromised plasma membranes (necrotic cells). Therefore these LDH assay results point to the high amount of necrotic cellular death. Moreover, the decrease in metabolic activity as measured is indicative of cell damage but does not indicate if necrotic or apoptotic death is occurring.

3.4.2 Effect of LSI/R on PPIase Activity

In healthy mitochondria, the inner membrane is impermeable to the majority of small molecules. This situation maintains the membrane potential as well as ensuring that there is a pH gradient necessary for the production of ATP during oxidative phosphorylation. The mitochondrion is one of the primary sites of damage in myocardial ischaemia reperfusion, forming the MPTP. This has been described by Bernardi and colleagues as a CsA sensitive, voltage dependent, high-conductance inner membrane channel,⁴⁶ It is induced by ROS and high concentrations of Ca^{2+} and phosphate as well as adenine nucleotide depletion. CyP-D located in the mitochondrial matrix, is recruited to a complex formed by VDAC and ANT, which assembles to form the MPTP in pathological conditions such as ischaemia/reperfusion injury. CyP-D, a member of the cyclophilin family, possesses the ability to catalyse protein conformation through its inherent *cis-trans* isomerisation activity. Upon binding to the ANT-VDAC complex, a conformational change is induced which is facilitated by the PPIase activity of CyP-D. As the level of ROS increases there is a parallel increase in binding of CyP-D to the ANT-VDAC complex. It may be possible to measure these changes by measuring PPIase activity. Initially when PPIase activity was measured concentrations that inhibited PPIase activity were toxic to cardiomyocytes after exposure to CsA and 6 hours LSI/R. Using 30nm CsA was found not to be toxic to cardiomyocytes over the experimental time course (Figure 3-6) and inhibited PPIase activity (Figure 3-7). Hence PPIase activity was measured in mitochondria (Figure 3-8), which were isolated at different time points.

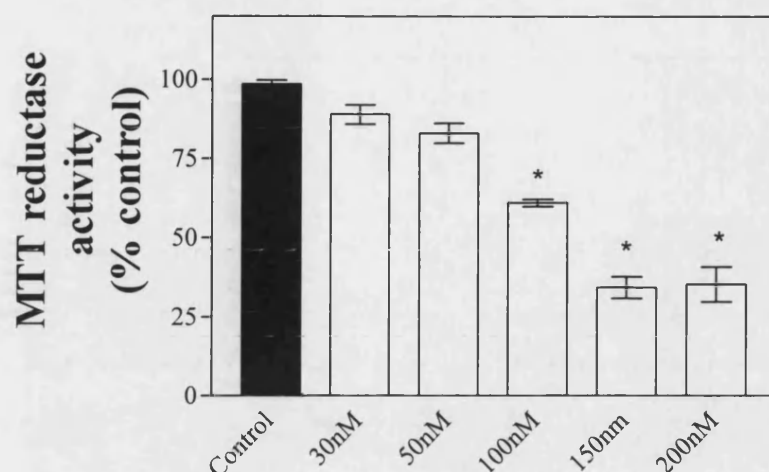


Figure 3-6. Protective curve for CsA treated cardiomyocytes prior to 3hrs LSI/R

Primary cardiomyocytes were incubated with 30nm cyclosporin CsA for 30 minutes and then subjected to 3 hours LSI with 24 hours reperfusion. Viability was assessed by MTT metabolic activity. 100nm, 150nm and 200nm samples are significantly different from control. Data are represented as the S.E.M of 4 individual experiments. * $P < 0.01$ vs control.

When CyP-D enzymatic activity was measured, no statistically significant difference in PPIase activity was found between any of the ischaemic/reperfused samples or between these and their respective controls (Figure 3-8).

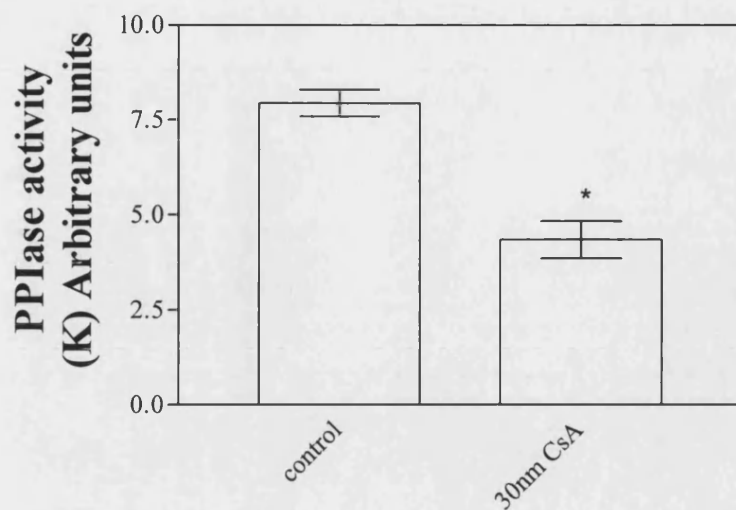


Figure 3-7. Reduction in PPIase activity as a result of CsA incubation

Neonatal cardiomyocytes were incubated with 30nm CsA for 30 minutes prior to analysis of PPIase activity. Reduction of PPIase activity by CsA is significant. Determination of PPIase activity arbitrary units (K) is described in section 2.2.1. Data are representative of the S.E.M of 3 individual experiments. *P<0.01 vs control

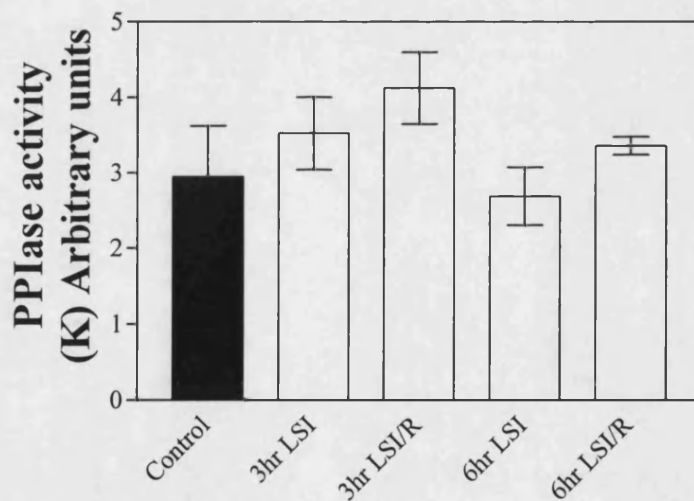


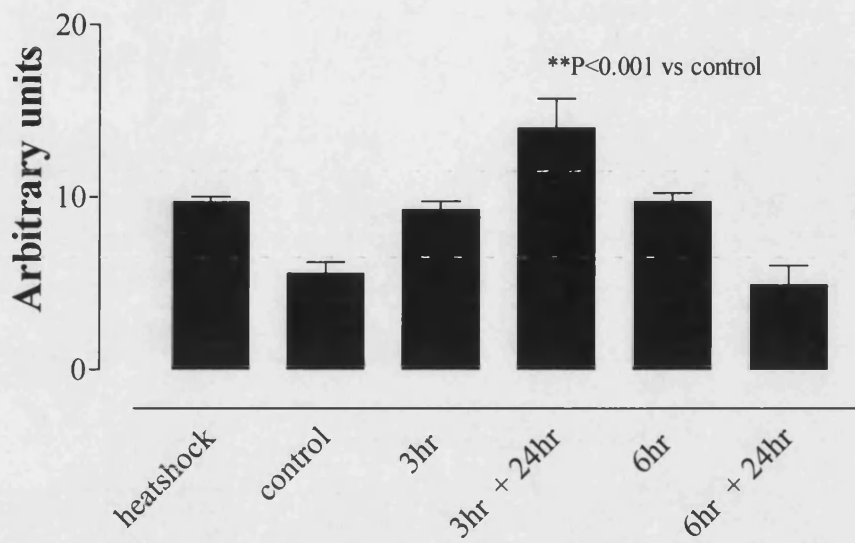
Figure 3-8. PPIase activity of isolated mitochondria

PPIase activity of mitochondria from cardiac myocytes that have been subjected to varying degrees of LSI/R. There was no significant statistical difference in activity between any of the different groups or between the respective groups and the control. The PPIase activity in each group was measured three times (mean ± SEM).

3.4.3 *Western Blot*

Because IR is reputed to involve the *de novo* synthesis of heat shock proteins⁸⁰ The synthesis of various HSP's such as HSP70 was examined. As is evident in Figure 3-9, of the expression of HSP70 at 24 hours reperfusion post LSI. Evaluation of CyP-D protein expression revealed no significant differences between any of the samples (Figure 3-10).

A



B

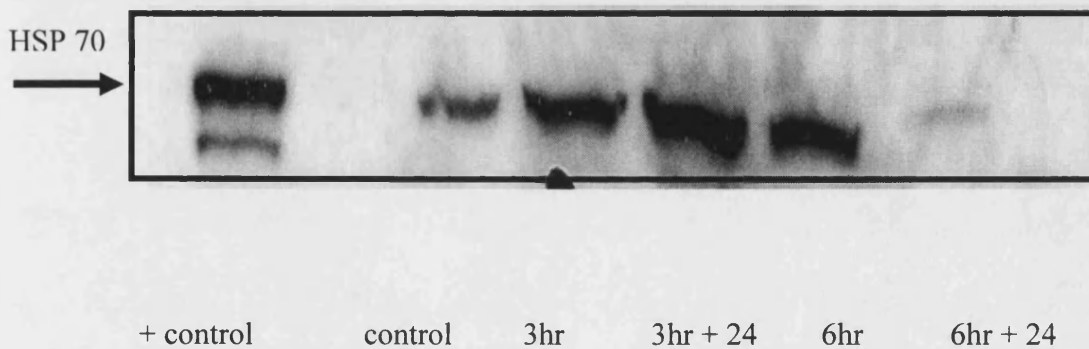


Figure 3-9 Cardiomyocyte protein expression of HSP70 after subjection to LSI/R

The Western blots for HSP70 expression in rat neonatal cardiomyocytes, subjected to differing degrees of LSI followed by reperfusion (B). This is also represented in graphical form (A) by measuring the density of the different bands using a densitometer as already described in Chapter 2. Data are representative of 3 individual experiments
 **P<0.001 vs control

Mitochondria are considered to be one of the primary sites⁴¹ of I/R injury, characterised by the loss of $\Delta\psi_m$ which features early in cell death.¹⁴⁹ The decrease of the $\Delta\psi_m$ was

measured by the fluorochrome DiOC₆ (3), a specific fluorescent cationic dye that is readily sequestered by active mitochondria depending on their $\Delta\psi_m$.

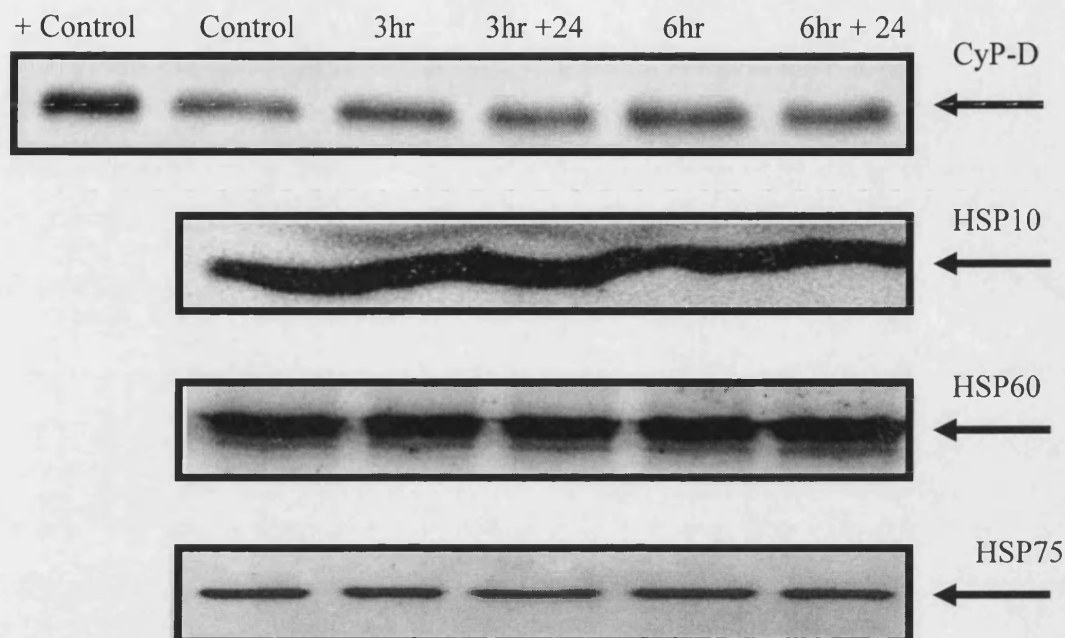


Figure 3-10. Western blot analysis of CyP-D, HSP10, HSP60 and HSP75 expression on cardiomyocytes.

LSI/R protein samples were electrophoresed on a 15% SDS-polyacrylamide gel. Following transfer they were probed for CyP-D, HSP10, HSP60 and HSP75 protein expression. LSI/R does not cause a significant change in CyP-D, HSP10, HSP60 and HSP75 expression

DiOC₆ (3) was used at a concentration of $<1\text{nm}$, to ensure that changes in the $\Delta\psi_m$ were being measured and not the plasma membrane.¹³⁶ As a further measure to ensure that the lack of change in $\Delta\psi_m$ was not due to any methodological problems, cardiomyocytes were incubated with differing concentrations of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) ranging from 0 to $500\mu\text{M}$ (Figure 3-11). This protonophore “short circuits” the respiratory chain by uncoupling respiration from oxidative phosphorylation which will abolish ATP synthesis collapsing the pmf and consequently $\Delta\psi_m$, independently of MPTP formation.^{150,151} Viability was also measured by the MTT assay as demonstrated in Figure 3-13. In Figure 3-14 a dot plot demonstrates the effect of various concentrations of CCCP on cardiomyocytes as

visualised on the FACscan flow cytometer and Figure 3-12 also demonstrates the shift in fluorescence caused by CCCP application as opposed to control samples.

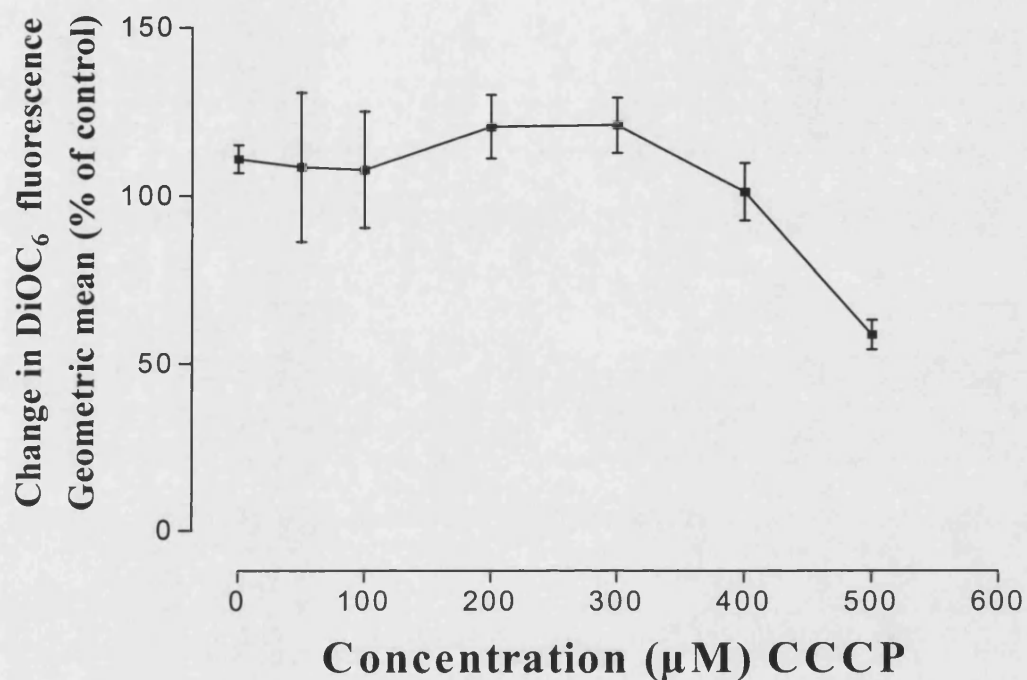


Figure 3-11. CCCP positive control

This reflects the decreases in $\Delta\psi_m$ in response to the uncoupler CCCP. The mitochondrial uncoupler CCCP attacks the mitochondrial electron transport chain resulting in the disruption of $\Delta\psi_m$. The disruption of $\Delta\psi_m$ can be measured with the lipophilic cationic dye DiOC₆ (3) manifesting itself as a reduction in fluorescence of the probe DiOC₆ (3) (reduction in geometric mean).

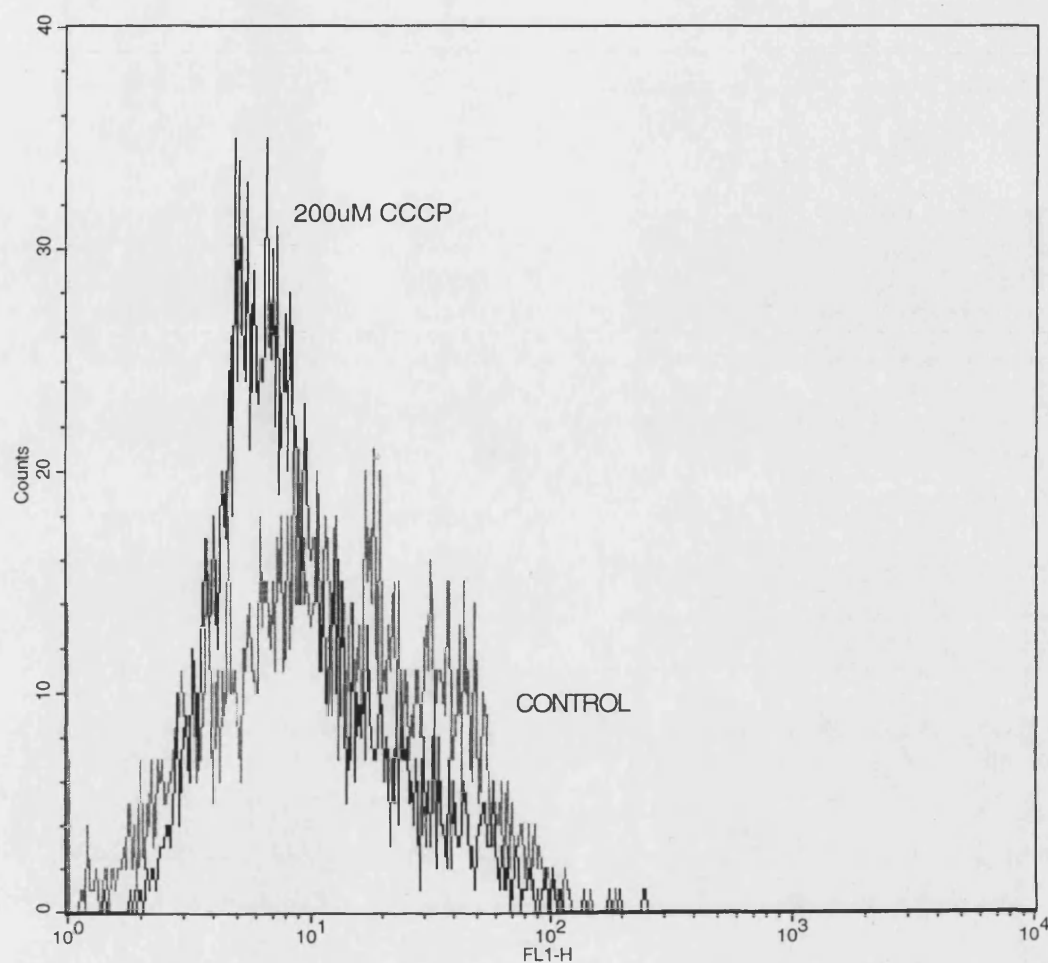


Figure 3-12. CCCP administration causes a shift in fluorescence

Figure 3-12 shows the effect of differing concentrations of CCCP on the $\Delta\psi_m$ as represented by the geometric mean using the fluorochrome DiOC₆ (3). The membrane shift is very clearly demonstrated as opposed to the control by the overlapped histograms. This trace shows the shift that occurs after application of 200 μ M CCCP. The abscissa displays the fluorescence intensity and the ordinate displays the number of cells.

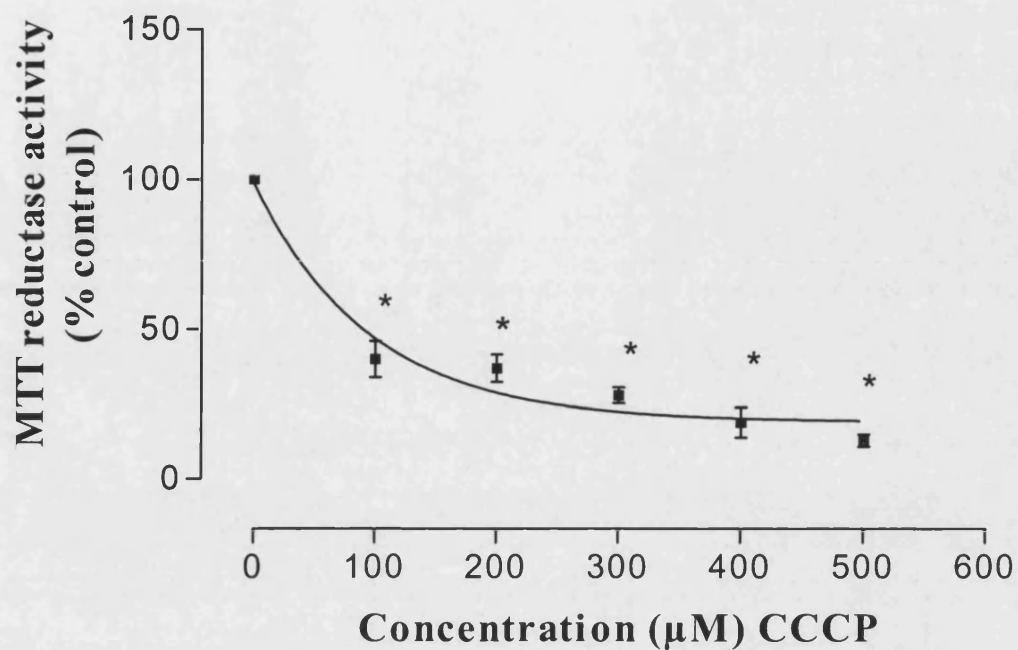
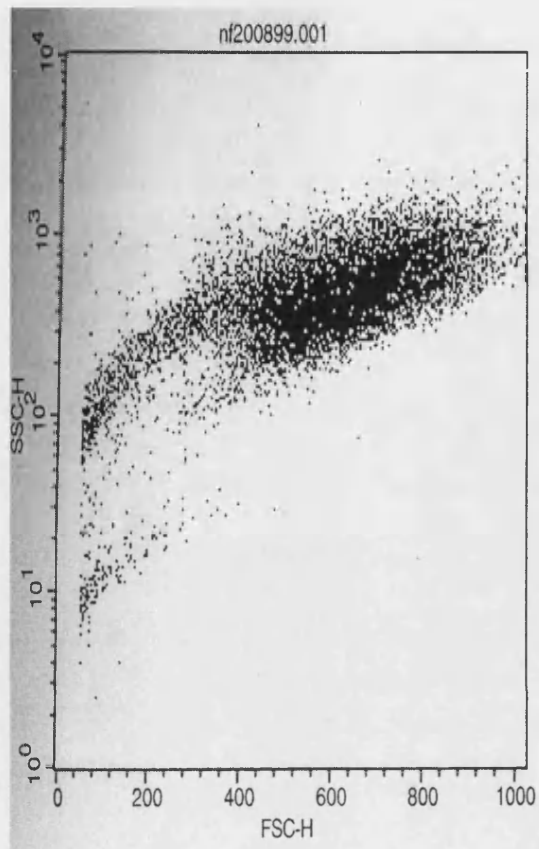


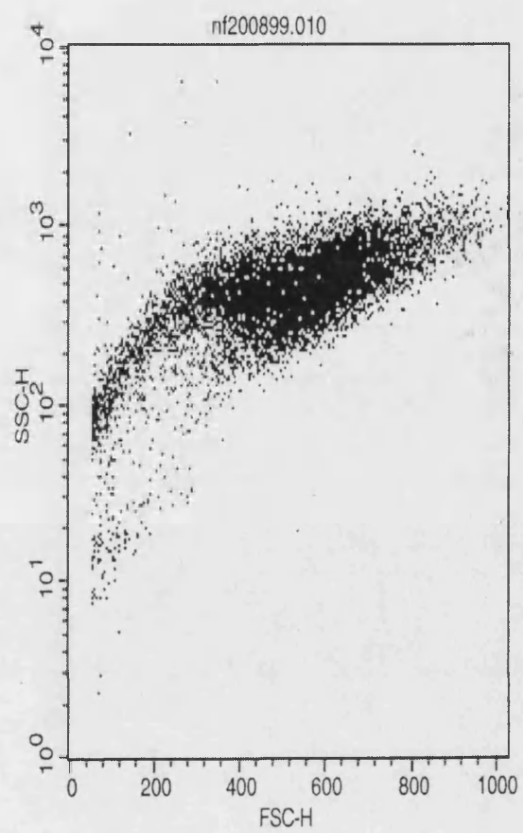
Figure 3-13. Survival of rat neonatal cardiomyocytes following exposure to CCCP.

Cardiomyocytes were exposed to varying concentrations of the mitochondrial uncoupler CCCP and survival rates were measured by MTT viability assay. Data is representative of the mean S.E.M \pm of at least three determinations for each experiment *P<0.001 v control

A control



B 100uM CCCP



C 300uM CCCP

D 500uM CCCP

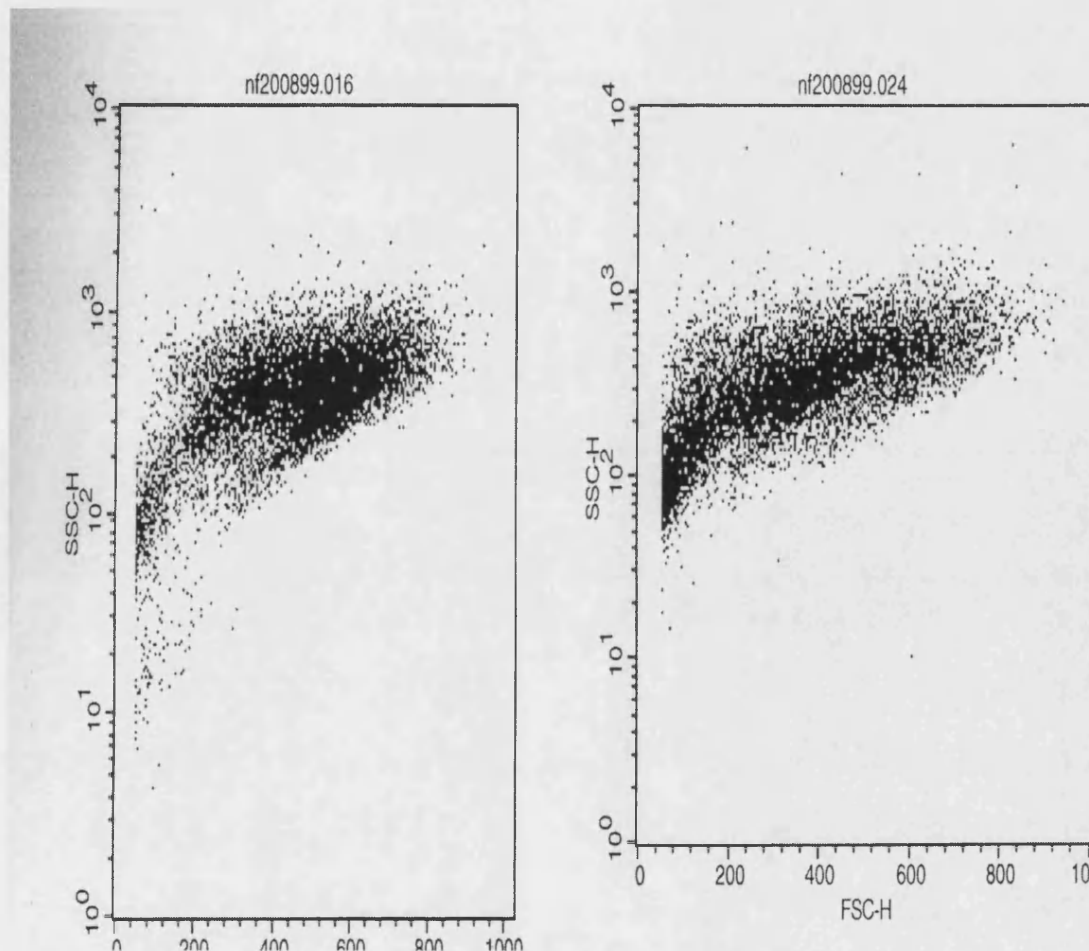


Figure 3-14. Representative diagrams demonstrating the shift in fluorescence.

This panel of diagrams in **Figure 3-14 (A, B, C, D)**, represent the effect of CCCP treatment on cardiomyocyte cells as visualised on the FACScan. The effect of increasing concentration of CCCP ranging from 200uM (**Figure 3-14A**) to 500uM (**Figure 3-14D**) is manifested as decrease in $\Delta\psi_m$ and physical shift of the cell population as CCCP uncouples the mitochondrial respiratory chain. The shift to the left occurs, as there is a decrease in size and granularity of the whole population, representing loss of viability. This shift to left of the population increases from **Figure 3-14 A** to **B, C** and finally in **Figure 3-14D**, the shift is dramatically different from the control (**Figure 3-14A**).

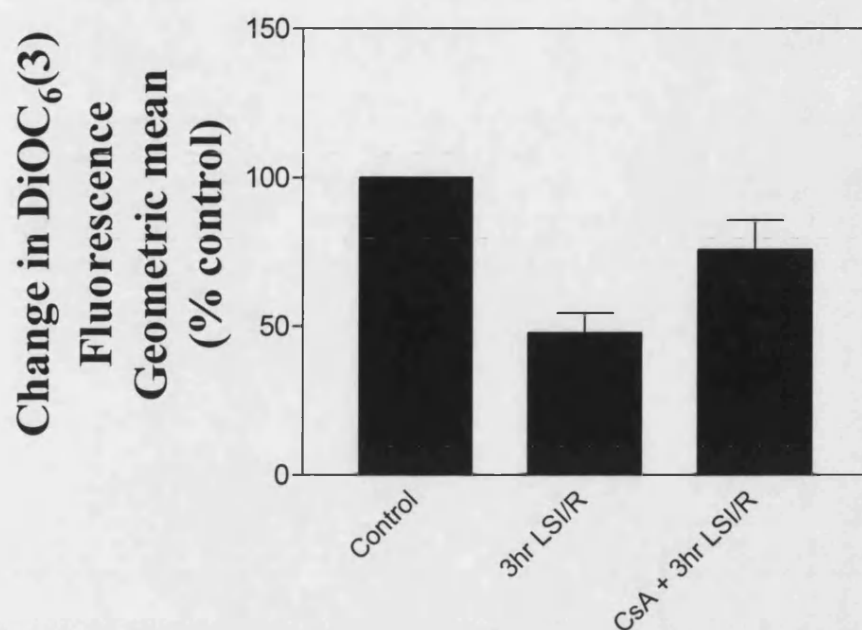


Figure 3-15 Cyclosporin A (CsA) protection against 3 hours LSI/R.

Graph demonstrating the effect of 3 hours LSI with followed by 24 hours reperfusion (LSI/R) with and without prior incubation with 30nm CsA as determined by the mitochondrial membrane potential dye, DiOC₆ (3). Protection afforded against 6 hours LSI/R is not statistically significant, but there is an increase suggesting a trend towards protection by CsA. Data are representative of means S.E.M of 3 individual experiments.

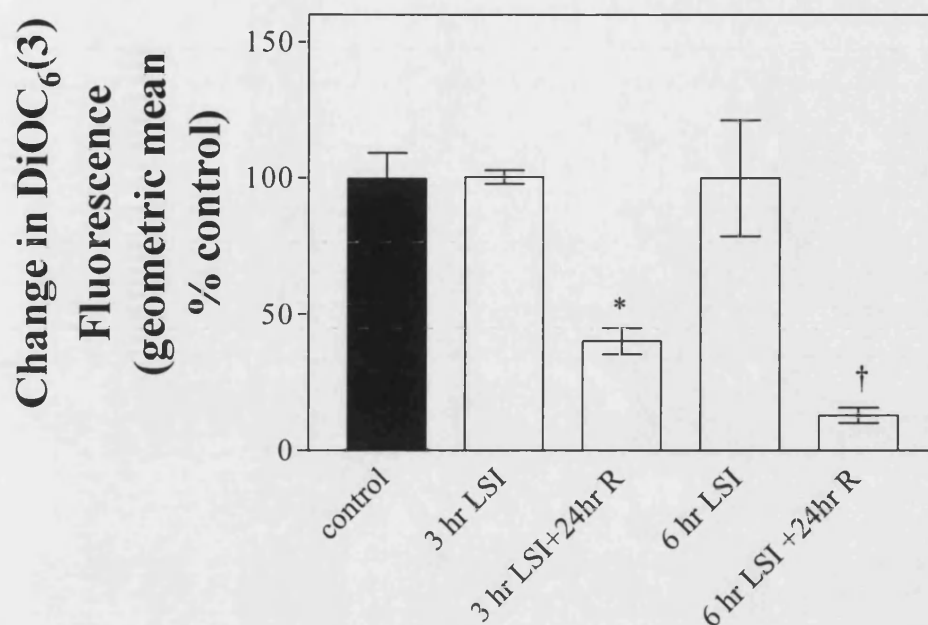


Figure 3-16. Graph displaying the effect of LSI/R on $\Delta\psi_m$ in cardiomyocytes.

The effect of increasing lethal simulated ischaemia/reperfusion (LSI) and reperfusion (R) on the $\Delta\psi_m$, which is represented as the geometric mean. Data are representative of the mean S.E.M \pm of 2 experiments performed in quadruplicate (n=4) *P<0.01, †P<0.001 vs control as analysed on the FACScan.

Figure 3-16 demonstrates the effect of LSI/R on $\Delta\psi_m$. It can be seen that 3 hours LSI does not affect mitochondrial membrane potential in comparison to the control, and 6 hours LSI does not differ significantly. Significant differences are demonstrated by cardiomyocytes, which have been subjected to 3 hours LSI with 24 hours reperfusion (LSI/R) and 6 hours LSI with 24 hours reperfusion (LSI/R). The $\Delta\psi_m$ decreases by approx 50% and 80% for 3 hours LSI/R and 6 hours LSI/R samples respectively in comparison to control levels. Figure 3-15 proves that the $\Delta\psi_m$ occurs as a result of the formation of the MPTP. In these experiments CsA is able to protect against the formation of MPTP and this is represented in Figure 3-15 by preservation of $\Delta\psi_m$.

3.4.4 *The Apoptotic and Necrotic Cell Death Ratio Resulting from LSI/R*

It is a well established fact that myocardial ischaemia reperfusion results in cell death. However there is much debate as to the significance of apoptosis in cardiac cells following ischaemia. It has been reported that global ischaemia results in mostly necrotic death in cardiac cells. The ratio is about 8: 92 of apoptotic to necrotic cells respectively.^{21,152,153} One of the reasons this LSI/R model was developed was to investigate whether cardiac cells died by apoptosis or necrosis in conditions which mimic myocardial IR. In order to achieve this, cardiomyocytes were subjected to 3 hr LSI and 24hr reperfusion as previously described. In addition another incubation time of 6hr LSI with 24 hr reperfusion was used. Immediately after the reperfusion period the supernatant from the culture vessels was spun down and prepared for electron microscopy analysis. The monolayer was removed and prepared for electron microscopy and a count was made on how many cells were apoptotic or necrotic. Identification of the cellular death types was based on the criteria listed below.

Morphological criteria of apoptosis were:

- Membrane blebbing, but no loss of integrity
- Aggregation of chromatin at the nuclear membrane
- Shrinkage of cytoplasm and condensation of nucleus
- Fragmentation of cells into smaller bodies
- Formation of membrane bound vesicles (apoptotic bodies)

Morphological criteria of necrosis were:

- Loss of membrane integrity
- Swelling of cytoplasm and mitochondria
- Disintegration (swelling) of organelles

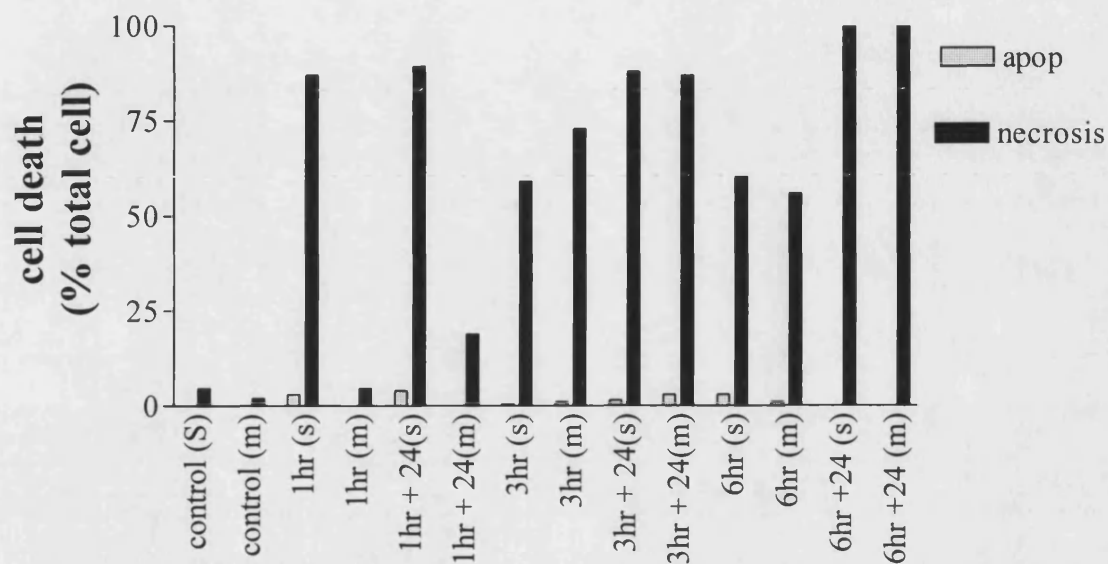


Figure 3-17. Apoptosis and necrosis post LSI/R.

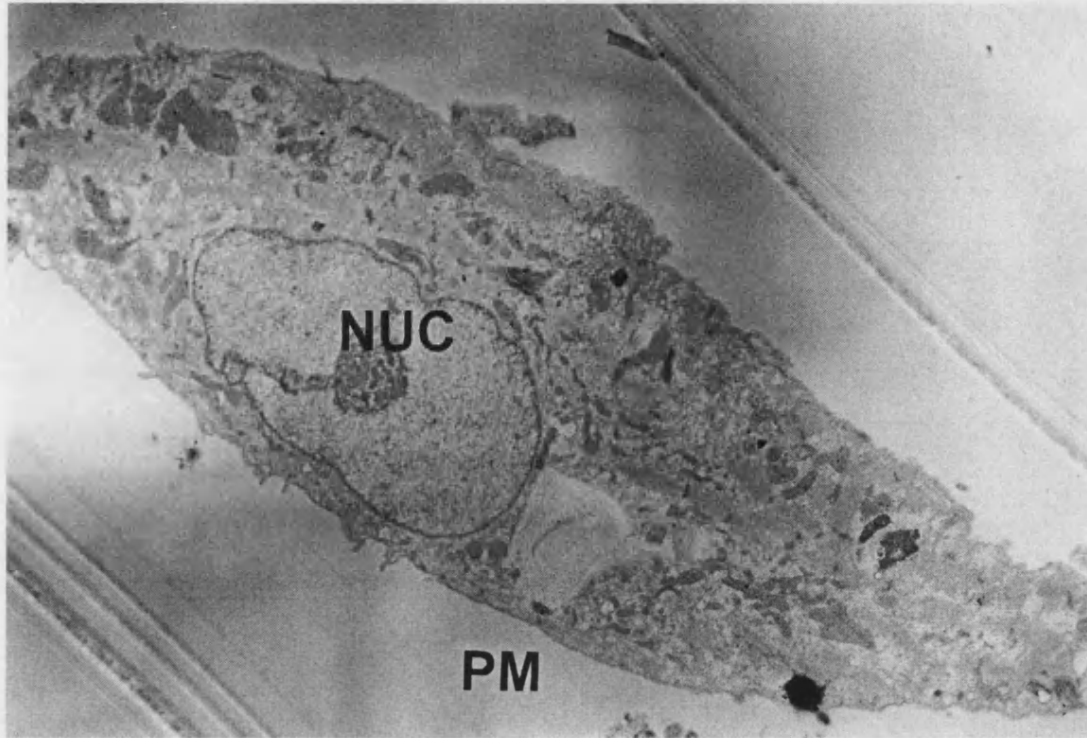
The effect of LSI on the rate of apoptotic and necrotic cell death was assessed in primary neonatal cardiomyocytes after 1, 3 and 6 hr lethal ischaemia followed by 24hr reperfusion. S and M represent cells that have been extracted from those floating in the supernatant and that are adherent on the monolayer respectively.

As was demonstrated in the above graph (Figure 3-17), there was minimal apoptosis present. There was no significant difference in the amount of apoptotic cells between each of the different treatments. The vast majority of cells died by necrosis ranging from 4 - 100% necrosis in the supernatant and monolayer of cardiomyocytes subjected to 1 - 6hr LSI alone and with 24hr reperfusion.

3.4.5 Ultrastructural Analysis of Cardiomyocytes after LSI/R

The following electron micrograph pictures show ultrastructural changes as they occurred in cardiomyocytes subjected to LSI/R at different incubation time points. In

the control (non-ischaemic) cells in Micrograph B, the plasma membrane was intact and the chromatin structure is evenly distributed with a prominent nucleus (NUC). All cell organelles are intact and mitochondria have double membranes.

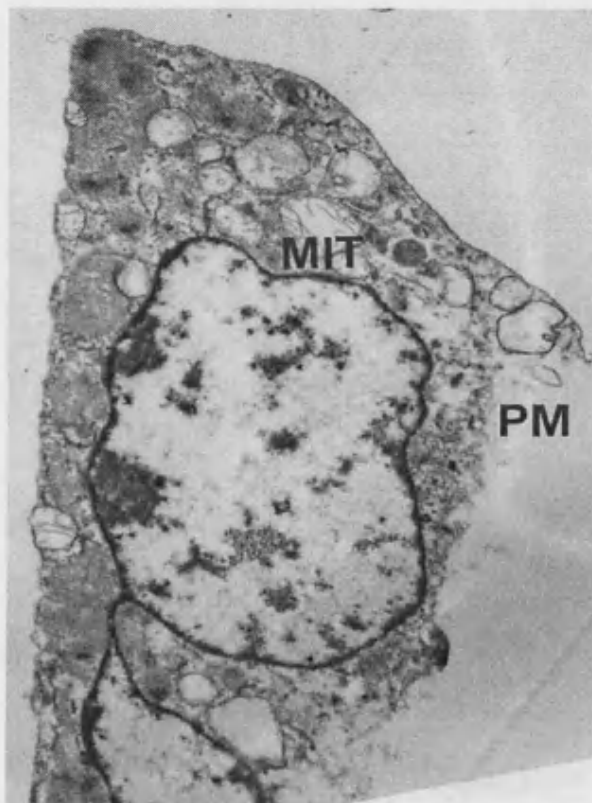


B

Micrograph B. Control myocytes

EM diagram of control myocytes, which demonstrate regular distribution of nuclear chromatin and an intact plasma membrane. The membrane is clearly visible in orderly rows. (Total magnification 5000x)

NUC=nucleus; PM= Plasma membrane



C



D

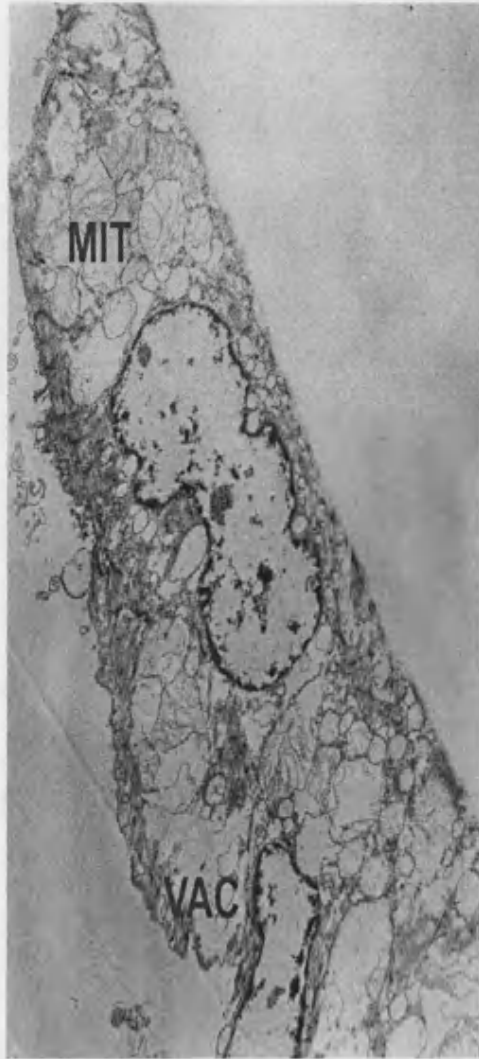
Micrograph C. Cardiomyocytes after 1 hour LSI

Swollen mitochondria, plasma membrane rupture and chromatin condensation.
MIT=mitochondria; PM= plasma membrane (5000x)

Micrograph D. Cardiomyocyte after 1 hour LSI + 24 hours reperfusion

Vacuoles are present in the cytoplasm. VAC=vacuoles (5000x)

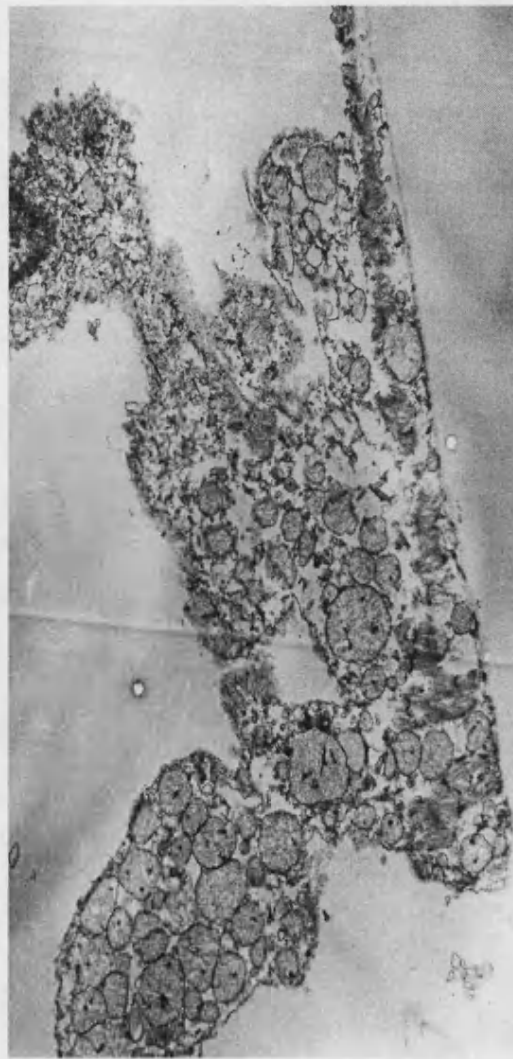
After 1 hour ischaemia and 0 hour reperfusion, we can observe that this cell is damaged due to the appearance of swollen mitochondria (MIT), though many cristae are still intact and there is some plasma membrane rupture (PM) in Micrograph C and some evidence of patchy chromatin condensation. In Micrograph D Cardiomyocytes have been subjected to 1 hr ischaemia and 24 hr reperfusion resulting in the appearance of some vacuoles (VAC).



E

Micrograph E. Cardiomyocytes subjected to 2 hours SI

Nuclear clumping, increased vacuolation and swollen mitochondria (5000x).
MIT=mitochondria; VAC=vacuolation

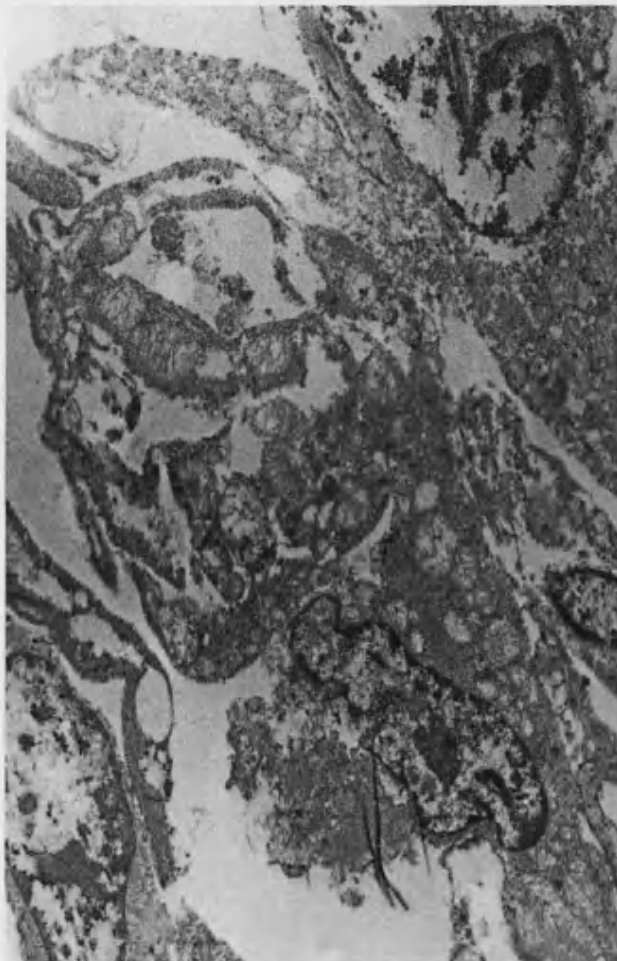


F

Micrograph F. Cardiomyocytes subjected to 2 hours LSI and 24 hours reperfusion

Increased damage but overall structure is still intact. (5000x)

Where cardiomyocytes have been subjected to 2 hour ischaemia and 0 hours reperfusion. Early signs of nuclear clumping, characteristic of necrosis are observed. The nucleus is intact and at this stage the damage is probably reversible (Micrograph E). In the subsequent Micrograph F in which cardiomyocytes have been subjected to 2 hours ischaemia and 24 hour reperfusion there is the appearance of vacuoles (VAC) but still overall cellular structure is intact.



G



H

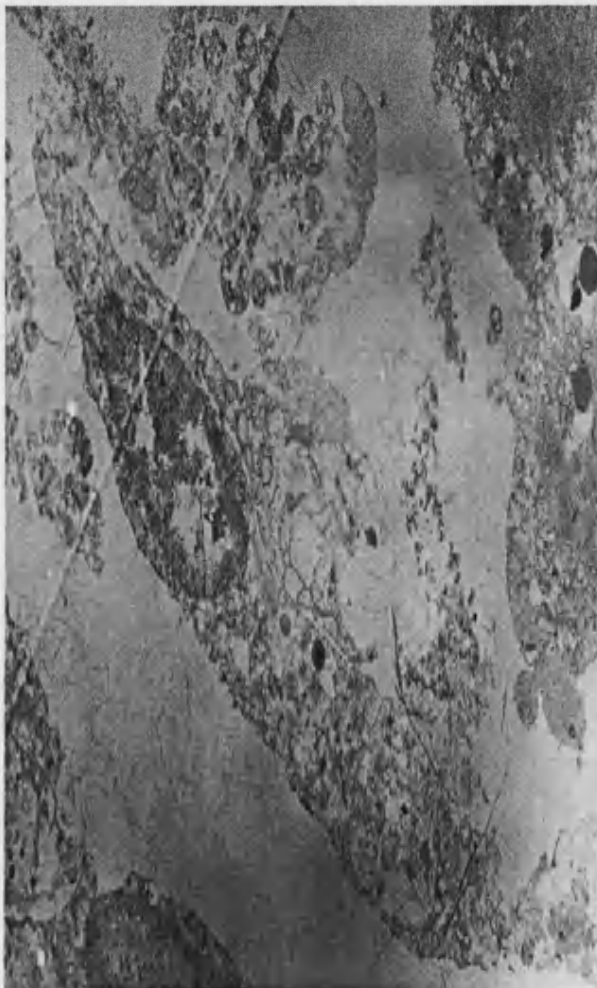
Micrograph G. Cardiomyocytes subjected to 3 hours LSI

Mitochondria are swollen and there is vacuolar formation (magnification 6000x)

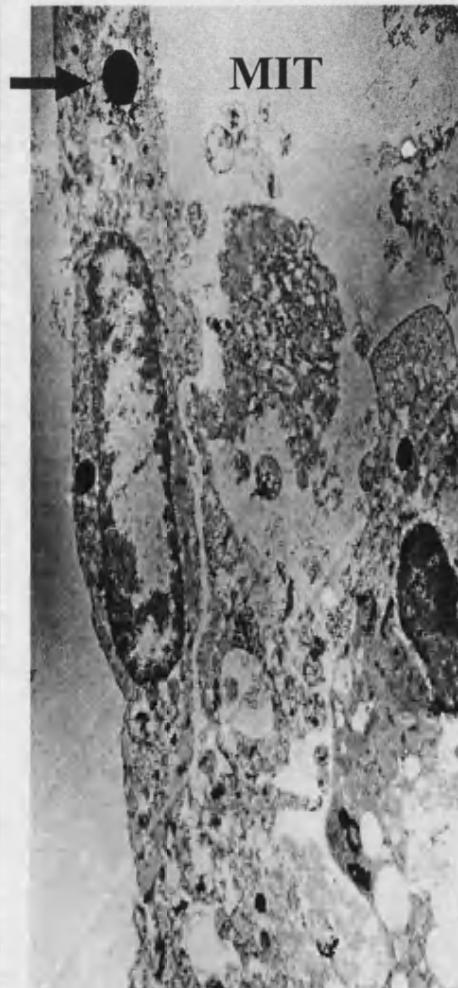
Micrograph H. Cardiomyocytes subjected to 3 hours LSI and 24 hours reperfusion

Nucleus has margined chromatin (arrow) and cytoplasm is shrunken, classic features of apoptosis (magnification 5000x).

In Micrograph G featuring cardiomyocytes subjected to 3 hours LSI and 0 hour reperfusion the mitochondria are distinctly swollen. Instead of regular stacking of cristae (inner mitochondrial membrane) many of the vacuoles present are caused by the large spaces that appear in the mitochondria, pushing the cristae apart. This is probably due to the accumulation of electrolytes and water that are early signs of damage to the enzymes of the membrane sodium pump. The beginnings of nuclear margination can be seen, but the plasma membrane is still intact and muscle fibres are still visible. Micrograph H displays cardiomyocytes subjected to 3 hours ischaemia and 24 hours reperfusion in which there is enhanced nuclear margination as indicated by the arrow and the cytoplasm has been severely reduced which are characteristic features of apoptosis.



I



J

Micrograph I. Cardiomyocytes subjected to 6 hours LSI

Plasma membrane damage, indicates that cell is severely damaged (magnification 6000x)

Micrograph J. Cardiomyocytes subjected to 6 hours LSI and 24 hours reperfusion

Secretory granules (arrow) present released mitochondria in the cytoplasm. MIT=mitochondria (magnification 6000x)

In the following diagram, Micrograph I, after 6hr ischaemia only, damage sustained is irreversible. Upon 24hr reperfusion later in Micrograph J, there is total disruption of the cell resulting in unrecognisable cell organelles as is evident in the photographs of cells subjected to 6hr ischaemia only (I), and 6 hours of ischaemia followed by 24 hour reperfusion (J). There is complete breakdown of the plasma membrane and spillage of cellular debris and cell organelles into the surrounding environment.

3.5 Discussion

The model of ischaemia reperfusion injury that was established in isolated rat neonatal cardiomyocytes, attempts to mimic the conditions heart cells experience during myocardial IR injury including depleted oxygen, substrate deprivation, hyperkalemia, acidosis and lactate accumulation.

In this model, it has been determined that the mode of cell death is predominantly necrotic. The number of cells undergoing necrosis increases with the extension of LSI/R.

Treatment of cardiomyocytes with LSI over a period of 3 hours ischaemia alone resulted in little change in LDH release, but a dramatic change occurred upon reperfusion. In contrast metabolic activity as determined by the MTT assay was severely decreased to 50% of the activity in control cells after 3 hours ischaemia alone and continued to decrease upon reperfusion. A sudden cessation in contraction on the addition of the ischaemic buffer was observed. This coincided with the instantaneous decrease of metabolic activity as determined by MTT assay. It was concluded that the

duration of ischaemia is an important factor in controlling whether or not cardiomyocytes initiate contraction upon reperfusion; for example after 3 hours ischaemia, cells would contract up to 48 hours after reperfusion, after 6 hours LSI/R they contracted for up to 12 hours only and after 18 hours LSI/R they would not contract at all when reperfused.

The main source of ROS in cardiomyocytes is the mitochondrial respiratory chain.^{41,50} Greater than 90% of total body oxygen is utilized by the mitochondrial respiratory electron transport chain¹⁹ of which 2% of the mitochondrial energy consumption results in production of ROS from complex 1 and 3. Ischaemia decreases mitochondrial antioxidant defence mechanisms such as MnSOD and glutathione peroxidase activity predisposing the mitochondrion to oxidative damage.¹⁵⁴ Mitochondria as well as being a source of ROS are also the target for the damaging effects of ROS.¹⁵⁴ This damage manifests itself in the formation of the MPTP with a concomitant decrease in the $\Delta\psi_m$.⁴⁶

As ischaemia progresses, inhibition of oxidative phosphorylation occurs, which causes ATP concentration to decrease. The ever decreasing ATP concentrations and increasing anaerobic glycolysis inhibit the Na^+/K^+ ATPase resulting in a moderate increase in Ca^{2+} .¹⁵⁵ Acidosis develops, which delays the onset of cell death,³⁸ and pore opening.⁵⁷ Upon reperfusion there is a much larger increase in intracellular Ca^{2+} . The formation of ROS increases, inducing oxidative stress and the pH returns to normal, which along with Ca^{2+} overload, act in concert to induce the onset of the MPT and a decrease in the mitochondrial membrane potential ($\Delta\psi_m$). This process is believed to occur as a consequence of reperfusion injury.¹⁵⁶ Controversy exists in regards to the use of lipophilic dyes to measure $\Delta\psi_m$ and whether collapse of $\Delta\psi_m$ is indicative of MPTP formation.¹⁵¹ Some researchers have developed models, which demonstrate that pore opening and collapse of the $\Delta\psi_m$ are interdependent.³⁸ However, it is generally agreed that maintenance of the $\Delta\psi_m$ is important in ensuring viability of the cell as mitochondrial diseases affect ATP production, and thus ultimately $\Delta\psi_m$ collapses.³¹ As collapse of $\Delta\psi_m$ is an early indicator of cell death, therefore in this study $\Delta\psi_m$ decrease in conjunction with survival assays are used to ascertain cell viability. Moreover, from the data of published reports, this study assumes or is based on the fact that the greater

the decrease in $\Delta\psi_m$ therefore the greater the likelihood that this is as a result of the opening of the MPTP.^{22,118} And as previously mentioned an uncoupler (CCCP) was used in this study to obtain depolarisation of $\Delta\psi_m$ and to confirm that the fluorescent probe DiOC₆ (3) can record this change. It has also been proved by the use of CsA that depolarisation of mitochondrial membrane potential measured by DiOC₆ (3) in this model relates to the formation of the MPTP.

In the present studies, ischaemia alone did not cause a decrease in $\Delta\psi_m$, only after reperfusion, which would suggest that the MPTP may have been formed after reperfusion, corresponding with previous reports by Halestrap and colleagues¹⁴⁰ as well as Duchen and colleagues.¹⁵⁶ By incubating in LSI only, there was no observable decrease of $\Delta\psi_m$ in this model.

There was no LDH release during ischaemia. However, we observed an increase in LDH release after 2 and 24 hours reperfusion and MTT activity was reduced by more than 50%. The decrease in metabolic activity was due to glycolysis inhibition and ATP decrease, which in turn may explain why the cardiomyocytes stopped contracting after addition of the ischaemic buffer. Soon after reperfusion metabolic activity continued to decrease, although some of the cardiomyocytes began to contract. A previous report has stated that heart muscle contraction occurs mainly during aerobic metabolism,⁴¹ which would explain the cessation in ischaemia and continuation of contraction after reperfusion. We have previously observed and it is well reported, that the duration of ischaemia determines viability of cells. With moderate ischaemia the cells recover when reperfused, but with severe ischaemia (in this model, 3 hours) cells may die¹⁵⁷ upon reperfusion. It is generally believed that the determining factors involved in this form of cell death are loss of ATP and adenine nucleotides, increase in intracellular Ca²⁺, and oxidative stress, all these events acting synergistically to provoke both necrotic and apoptotic cell death.^{38,41,157,158}

Reports from other centres relating to the presence of apoptosis in IR injury are quite divergent. In 1994 Gottlieb and colleagues¹⁵⁹ initially described the occurrence of apoptosis in rabbit hearts, which had been subjected to IR damage. In contrast, a

Japanese group reported that in their experiments with rabbit hearts subjected to coronary artery occlusion, EM analysis revealed no signs of apoptosis. Taimor and colleagues found using a simulated ischaemic (SI) model of adult cardiomyocytes, that they could not induce apoptosis. Increasing SI only resulted in a greater degree of necrotic cells.¹⁶⁰ They speculated that the lack of apoptosis was due to the fact that exogenous sources of ROS in ischaemically reperfused myocardium may contribute to the induction of apoptosis. *In vivo* the exogenous ROS might originate from other cell types such as neutrophils. In their model (as in the model in this study) single cells populations were used (cardiomyocytes) and therefore no exogenous sources of ROS were present. Taimor and colleagues used the same model again but using H₂O₂ as exogenous sources of ROS caused apoptosis in another study.¹⁶¹

Using our model of LSI/R, we investigated which type of cell death pathway predominated, whilst subjecting cells to differing periods of ischaemia and reperfusion. Using transmission electron microscopy (TEM) it was possible to differentiate between apoptotic and necrotic cells during the different ischaemic and reperfusion time points. We observed that very little apoptotic cell death occurred during the shortest ischaemic and reperfusion time points. As the duration of ischaemia progressed from 1 to 6 hours, the rate of necrotic cell death increased to 100%. The LDH assay, which measures enzyme release from cells with compromised plasma membranes, in other words necrotic cells, correlated with the TEM data. Decrease of metabolic activity was registered with prolonged LSI/R.

Electron microscopy has been described as “the gold standard in identifying apoptosis and necrosis.”¹⁶² Nevertheless, some authors disagree and argue that this method is too labour intensive and prone to “subjective interpretation of morphological findings.”¹⁶³ They instead recommend the use of terminal deoxynucleotidyl transferase (TdT) mediated dUTP-biotin nick end labelling (TUNEL). This assay detects single and double stranded DNA breaks with 3' OH termini and therefore TUNEL may not be so specific for the detection of apoptosis as some necrotic cells could also be TUNEL-positive.¹⁶⁴ Ohno and colleagues have used electron microscopy to analyse plasma membrane integrity coupled with TUNEL (EM-TUNEL) and they identified TUNEL

positive cells with compromised membranes. A few years earlier, Majno and Joris described myocardial cell death occurring by apoptosis, oncosis and necrosis (the final steps in oncotic and apoptotic cell death). Oncosis is accidental cell death which features “classical” cell swelling and membrane rupture.¹⁶⁵ In the same vein, Anversa has classified myocardial cell death as necrosis, apoptosis and necrosis-apoptosis, the latter being a form of cell death with common features.¹⁶³ This type of “shared” features in cell death is mentioned by other authors.¹⁶⁶ Apoptosis is an energy requiring process; however, during apoptosis if energy levels fall necrosis ensues and hence the phenomenon is commonly known as secondary necrosis occurs. Cell death can not be solely defined as one or the other, apoptotic and necrotic pathways are becoming quite blurred and so another type of cell death name was coined, necrapoptosis. This term represents the shared characteristic form of both apoptosis and necrosis.¹⁶⁶

Ischaemia, heavy metals and other pathological stresses induce the production of cytoprotective stress or heat shock proteins (HSP).^{58,65,147} The relationship between duration of LSI/R, heat shock protein expression and cytoprotection was analysed. This was achieved by screening for induction of HSP10, CyP-D, HSP60 and HSP70 using mono- and polyclonal antibodies for Western blotting. An induction in HSP70 expression was detected after 3 and 6 hours ischaemia with 0 hour's reperfusion. Upon reperfusion an increase in HSP70 expression reached a maximum in cells subjected to 3 hours LSI and 24 hours reperfusion only. Many investigators have stated that upregulation of HSP70 expression confers cytoprotection against a stress induced lethal injury.¹⁶⁷⁻¹⁷⁰

As well as using Western blotting to examine CyP-D expression, PPIase activity measurements did not reveal any differences. It has been reported that increased binding of CyP-D to the ANT-VDAC complex is partially caused by oxidative stress.¹⁷¹ We proposed that this might manifest itself as a change in PPIase activity or protein expression. However the results in this study have demonstrated that this increased binding was not manifested as an alteration in any of these factors. In this study the demarcation between apoptosis and necrosis were assumed to be distinctly different

with the methods used as described. Other researchers corroborate the results that have been obtained,¹⁶⁰ but this model has its limitations especially in the methods used for analysis of cell death. No biochemical assay to discern apoptosis and necrosis were used in this model. This model also does not take into consideration that apoptotic myocytes *in vivo* are phagocytised, and therefore in this model they may have developed necrotic features as a function of secondary necrosis. The use of TUNEL labelling in combination with the other methods used, may have altered the apoptotic count. The use of TUNEL in combination with TEM¹²⁵ would have enabled the identification of so called necrapoptotic cells. There is no doubt that using these methods would have helped to give a more accurate idea as to the processes occurring in this model and therefore add more strength to our argument and possibly raise a few more questions, however, the overall results probably would not have changed.

3.6 Conclusion

The aim of this present study was to develop a model using neonatal rat cardiomyocytes to investigate the effects of LSI/R on parameters such as mode of cell death, $\Delta\psi_m$, HSP expression, PPIase activity. HSP70 expression increased in relation to duration of LSI/R and was substantially reduced after 6 hours ischaemia and 24 hours reperfusion. $\Delta\psi_m$ decreased relative to the increasing duration of LSI/R. This study has shown that rat neonatal cardiomyocytes subjected to LSI/R die predominantly by necrosis with minimal apoptosis occurring. This is in agreement with a previous study¹⁶⁰ in adult rat cardiomyocytes using a similar ischaemic buffer. Over the years many investigators have supposedly demonstrated the occurrence of apoptosis in the heart. However the percentage of apoptosis occurring following myocardial infarction in IR is highly controversial. This is partly due to the problems associated with false positives associated with techniques such as TUNEL labelling of double stranded DNA breaks. Some groups have speculated that there are apoptotic-inducing factors released from exogenous blood or non-myocyte cells in ischaemically reperfused tissue. It also is thought that apoptosis may be induced in cardiomyocytes, as a result of sublethal injury

when mitochondria are in slight energy depleted state.¹⁷² Their report demonstrated that slight metabolic depression due to ischaemia/reperfusion results in apoptosis, whilst severe metabolic depression due to prolonged ischaemia /reperfusion causes mostly necrotic cell death.

In summary this present study demonstrates that as the duration of LSI/R increases apoptosis decreases until cell death is necrotic as determined by the survival assay employed and ultrastructural analysis. Elevation of HSP70 protein expression reached a peak at 3 hours LSI and 24 hours reperfusion and no significant increase in PPIase activity or expression of cyclophilin was observed.

CHAPTER 4

4. ISCHAEMIC PRECONDITIONING OF NEONATAL RAT CARDIOMYOCYTES WITH SI/R

4.1 Introduction

Ischaemic preconditioning (IPC) describes the phenomenon whereby a single or series of sublethal ischaemic stresses with intervening reperfusion enhances the tolerance of the myocardium to a more subsequent lethal ischaemia/reperfusion stress. This endogenous protective mechanism was first described in 1986 by Murry and colleagues⁷² in a canine heart model. Dogs were anaesthetized and subjected to four sequential 5-minute periods of regional ischaemia, each followed by 5 minute reperfusion, before a sustained 40 minute ischaemic insult. They found that brief periods of ischaemia did not cause necrosis but reduced the depletion of ATP. This preconditioning phenomenon since then has been demonstrated in many species such as rat^{77,173,174} pig¹⁷⁵ and rabbit^{80,95,96,176} as well as several organs such as liver⁷⁷ and brain.^{141,174} IPC in the myocardium occurs in two distinct phases: early, (acute or classical) preconditioning which starts immediately after the initial trigger and lasts for 2 - 3 hours, and a second window of protection, termed late or delayed preconditioning, occurs 12 to 24 hours after the initial stimulus lasting up to 3 - 4 days which is not as potent as classical preconditioning.^{71,80,177-179} This innate protection can enhance or restore contractile function of post ischaemic myocardium and protect skeletal muscle^{180,181} as well as reduce ATP depletion in the canine (preconditioned) myocardium⁷² subjected to a sustained ischaemic insult. The interest generated in such a powerful phenomenon has led to the drive to investigate the mechanisms of IPC, which in turn have helped to achieve a better understanding of the mechanisms involved

in ischaemia/reperfusion injury. It has been demonstrated that adenosine, bradykinin, noradrenaline, prostanoids, free radicals and nitric oxide (NO) are released from the myocardium¹⁸²⁻¹⁸⁴ of which some, but not all are believed to lead to the opening of K_{ATP} channels.^{183,185} By the nature of the different methods of preconditioning, it seems that different induction mechanisms are involved.

Adenosine is a breakdown product of ATP, which occurs, in high concentrations in ischaemic tissue.^{77,186} Adenosine is an initiator of preconditioning and is necessary for the mediation of preconditioning effects.¹⁸¹ The binding of adenosine can be blocked by selective A1 or A3 receptor antagonists, which lead to the inhibition of protection and therefore confirm the receptor-mediated nature of preconditioning.¹⁸³ Once the adenosine receptors have been activated, G-proteins coupled to these receptors are also activated.⁷⁷ This in turn, results in the activation of phospholipase C and DAG production followed by activation of membrane-associated protein kinase C, thereby inducing the translocation of specific PKC isoforms from an inactive cytosolic pool to the cell membrane. This has been proposed as a fundamental component of the PC mechanism.⁷¹ As K_{ATP} channels are linked to G-proteins, it has been suggested that they play a part in acute preconditioning responses. This is confirmed by the pharmacological action of K_{ATP} channel antagonists, which abolish the preconditioning response.^{71,183}

We investigated the existence of the delayed preconditioning phenomenon in primary cardiomyocytes. We also studied the correlation between cyclophilin enzymatic activities and delayed preconditioning as well as the expression of HSP's in these preconditioned cells.

4.2 Methods

4.2.1 Cell Culture

Primary neonatal cardiomyocytes were isolated as previously described in Chapter 2.

4.2.2 Ischaemic Preconditioning Protocol

The cardiomyocytes that had been seeded into 6 or 12 well plates were subjected to 1 hour simulated ischaemia (SI), reperused for 24 hours and then subjected to 3 hours lethal simulated ischaemia (LSI) and reperused for 24 hours (LSI/R) as indicated in the following diagram (Figure 4-1). The control cells in contrast were kept in maintenance media during the preconditioning process prior to subjection to LSI for 3 hours.

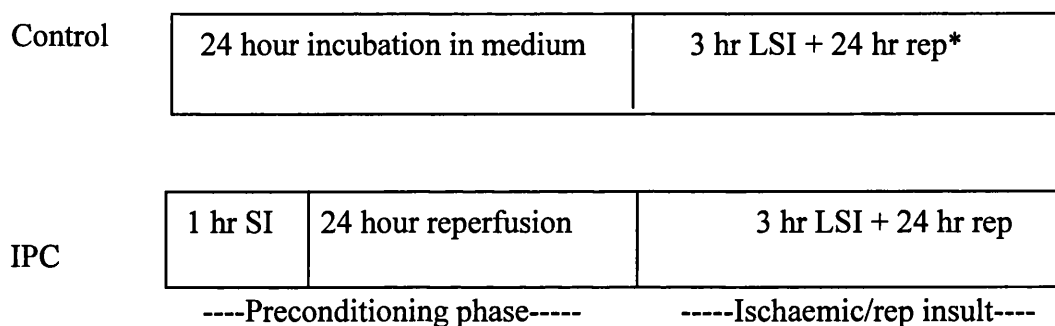


Figure 4-1. This is a description of the IPC protocol used in cardiomyocytes

*rep= reperfusion. Control cells were maintained in maintenance medium prior to subjection to the ischaemic/reperfusion insult. In contrast another group of cardiomyocytes were subjected to 1 hour simulated ischaemia (SI) and reperused for 24 hours. This was the preconditioning stimuli required, prior to exposure to the ischaemic/reperfusion insult.

4.2.3 Assessment of Viability, Protein Expression and PPIase Activity

After the end of 3 hours LSI + 24 hour reperfusion (LSI/R), some of the buffer was collected and the lactate dehydrogenase release from the cardiomyocytes was determined as described in Chapter 2. Metabolic activity was measured using the MTT assay also as previously described. Protein was extracted and Western blot analysis was performed in order to determine of heat shock protein expression. Cells were also collected for the PPIase enzymatic activity assay, which was described in Chapter 2.

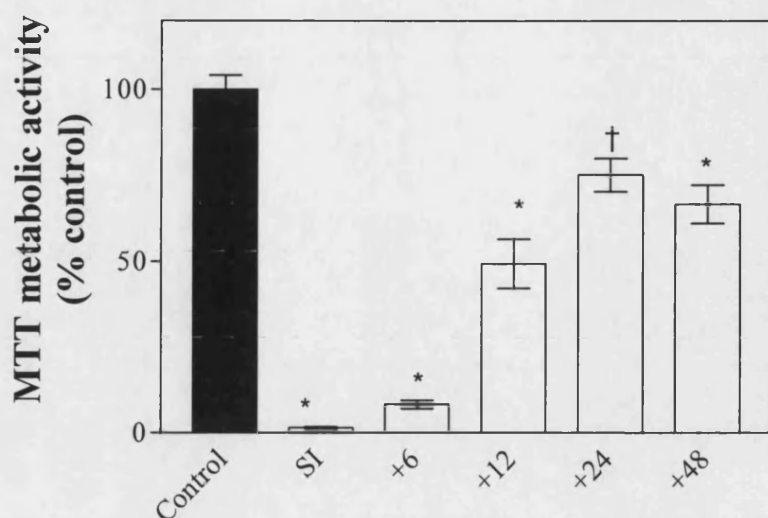
4.2.4 *Measurement of Mitochondrial Membrane Potential ($\Delta\psi_m$)*

Cells were preconditioned as described above and then subjected to LSI/R. Controls were incubated in media alone and then subjected to LSI/R. After these incubations cells were collected and stained with DiOC₆ (3) and the change in DiOC₆ (3) fluorescence was measured as previously described in Chapter 2.

4.3 **Results**

4.3.1 *Determination of Protection Afforded by Various Preconditioning Protocols*

Cardiomyocytes were initially subjected to 1hr SI and reperused for different periods. Then each differently reperused group of myocytes were all exposed to 3 hr LSI with 24 hr reperfusion (LSI/R) as a lethal insult. The viability of each of these differentially reperused cardiomyocytes was assessed by the MTT assay. This assay demonstrated which group of cardiomyocytes better survived LSI/R and was therefore ischaemically preconditioned. The graph in Figure 4-2 demonstrates that 1hr SI with 24hr reperfusion afforded the best protection against LSI/R. Consequently 1hr SI with 24hr was then used to precondition the cardiomyocytes in all of the following experiments.



Reperfusion Time after Simulated Ischaemia (hr)

Figure 4-2. Viability of Cardiomyocytes following 1 hour SI and different reperfusion time points

Each of these samples were initially incubated in 1 hour SI buffer and differing reperfusion time points, followed by subjection to LSI/R, then metabolic activity was measured in order to ascertain which time point afforded the best protection. These values are representative of means \pm SEM of 6 different preparations. * $P < 0.001$, † $P < 0.01$ v control.

4.4 Effect of IPC on Cell Survival against LSI/R

The degree of protection reflected in LDH release, in the preconditioned cells in comparison with non-preconditioned cells subjected to LSI/R was 30%. In comparison to the preconditioned and non-preconditioned cells, the difference in LDH release between these and the control was 40% and 30% respectively (Figure 4-3A). The MTT assay also demonstrated the increased level of metabolic activity in preconditioned cells in comparison with non-preconditioned cells, however the difference was only approximately 20% (Figure 4-3B). We also observed the effect of preconditioned and non-preconditioned cells on mitochondrial membrane potential. As is shown in Figure

4-4, LSI/R significantly decreased DiOC₆ (3) fluorescence and therefore decreased the $\Delta\psi_m$ in non-preconditioned cardiomyocytes, but preconditioned cells maintained a significantly high $\Delta\psi_m$ ($p<0.001$) after subjection to LSI/R. In preconditioned cells, contractile activity returned after reperfusion was established as visualised by the light microscope, but the same was not observed in non- preconditioned cells.

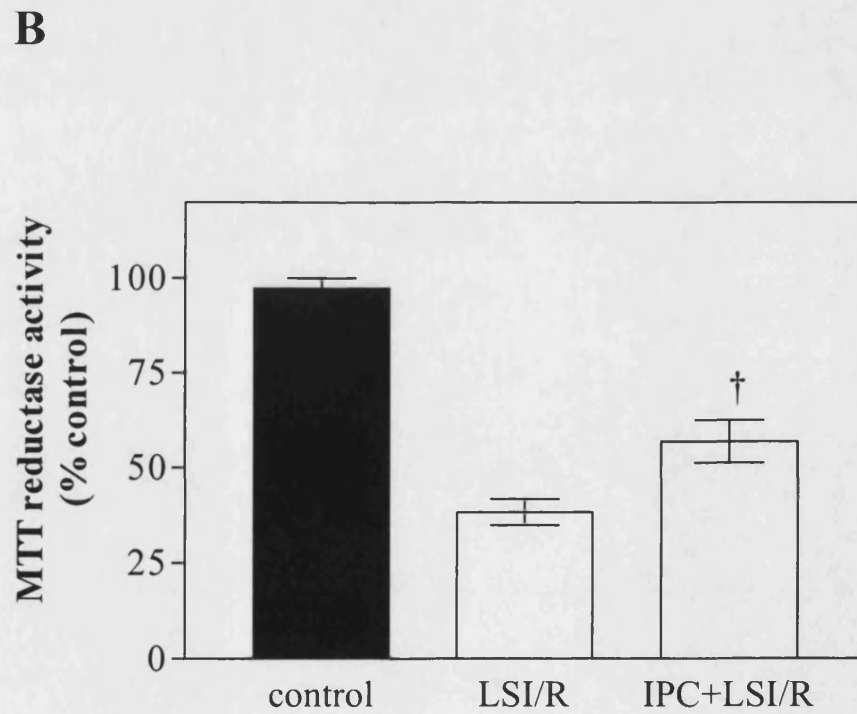
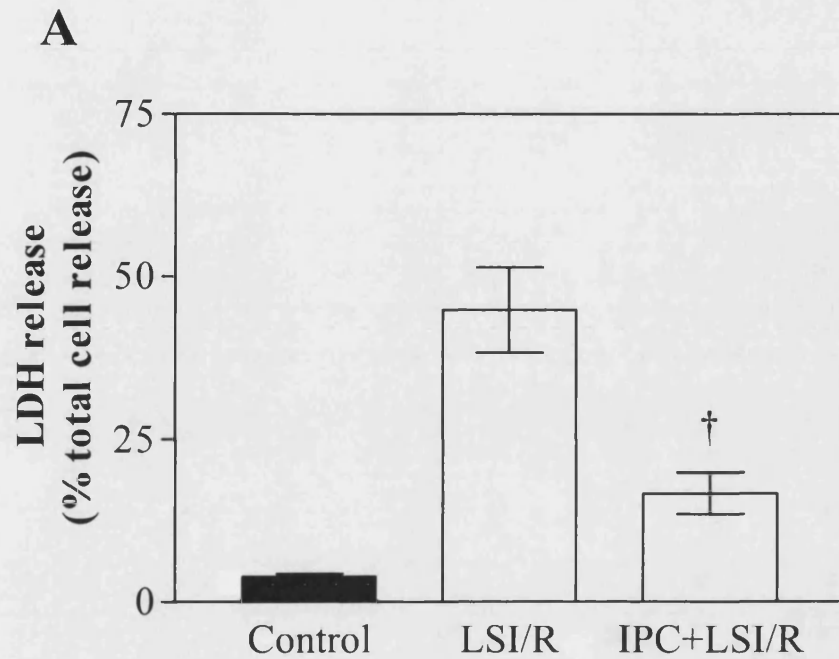


Figure 4-3. Effect of IPC cardiomyocytes after exposure to LSI/R

Demonstration of the protection afforded to preconditioned primary cardiomyocytes against non- preconditioned cells that have been subjected to 3 hour LSI/R as

determined by (A) LDH release and (B) MTT cell metabolic assays. In both graphs protection of IPC treated cells is significant ($\dagger P < 0.01$) for IPC samples after subjection to LSI/R for both assays. These results are representative of the means \pm SEM from 3 independent experiments ($n=18$). $\dagger P < 0.01$ vs LSI/R

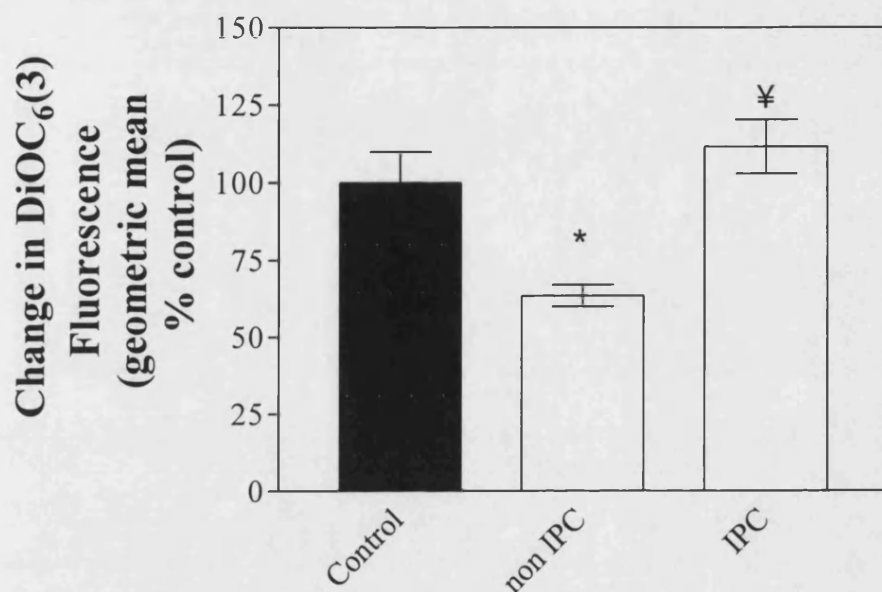


Figure 4-4. Effect of LSI/R on $\Delta\psi_m$ in non-IPC and IPC treated cardiomyocytes.

This graph demonstrates the significant protection ($P < 0.001$) afforded to IPC treated cardiomyocytes in comparison to non-IPC treated cardiomyocytes after subjection to LSI/R. These results are representative of the means \pm S.E.M of 3 independent experiments ($n=9$) for each sample and $*P < 0.01$ compared with control and $¥P < 0.001$ compared with non-IPC.

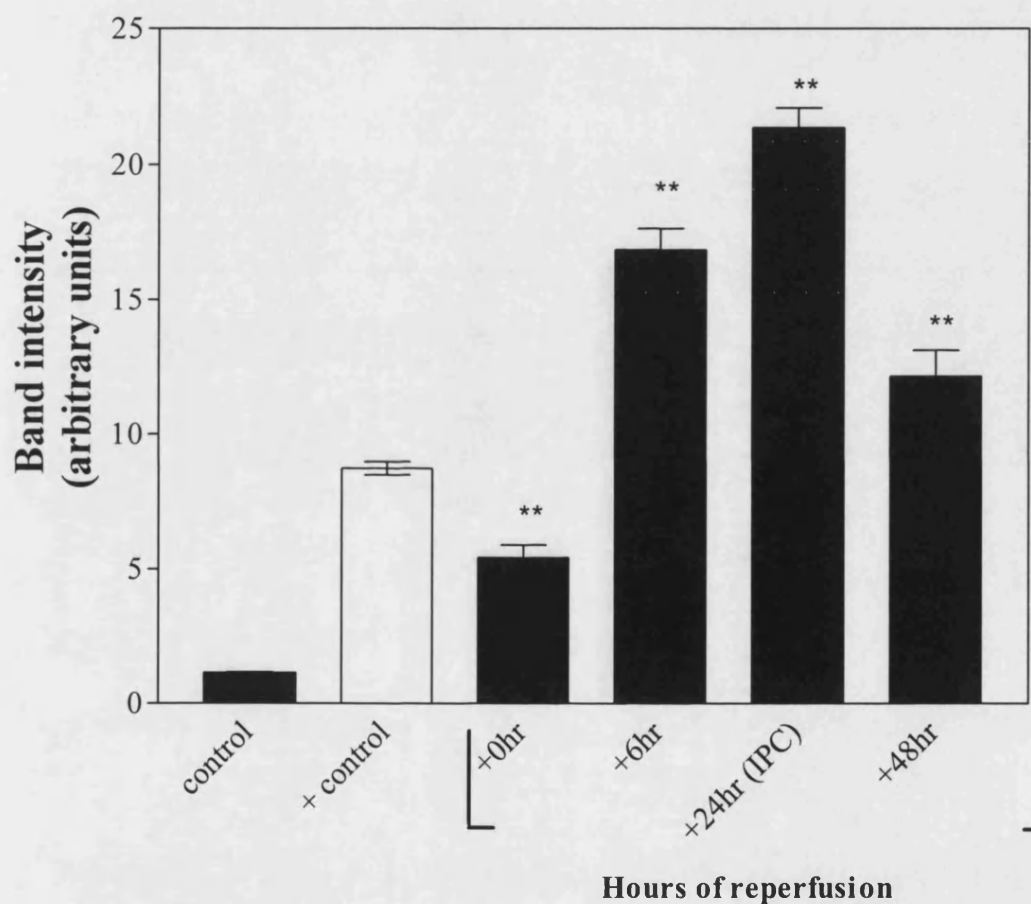


Figure 4-5 Graphic representation of HSP70 protein expression.

This graph reflects different levels of HSP70 expression in non-preconditioned and preconditioned cells and is a graphic representation of the Western blot below. The positive control (+ control) is a heat-shocked sample. The control is non-IPC treated cardiomyocytes and the +24 hour (IPC) sample is representative of ischaemically preconditioned cardiomyocytes with an increased expression of HSP70. Data are representative of mean S.E.M \pm 3 individual experiments **P<0.001 vs control

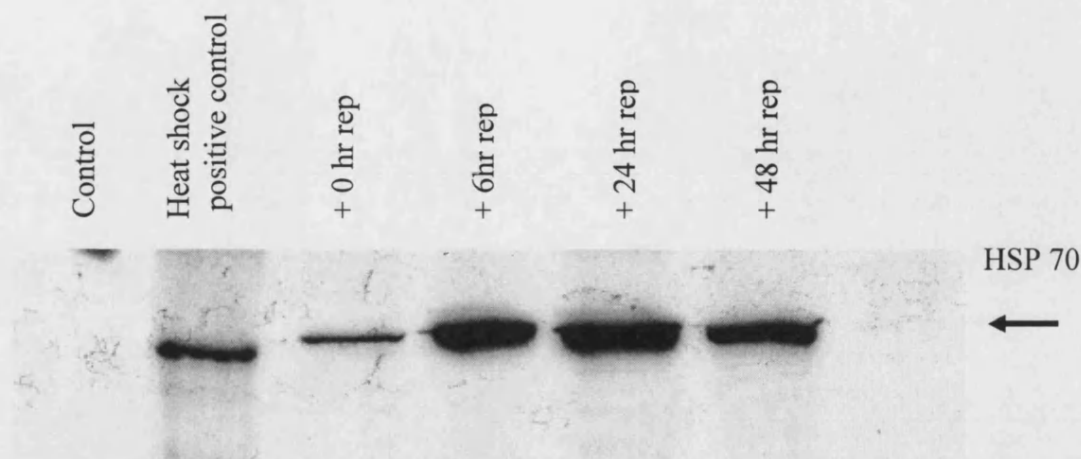


Figure 4-6 Western blot of HSP70 expression.

Effect of preconditioning stimuli on HSP70 expression in primary neonatal cardiomyocytes. Protein lysates were isolated after each of the reperfusion intervals and electrophoretically separated on a 12% SDS/PAGE gel. The individual bands were analysed on a densitometer and the above graph was constructed from the results.

The current preconditioning protocol was determined by increased viability of cells when subjected to LSI/R as opposed to non-preconditioned cells subjected to LSI/R. We analysed whether any of the heat shock proteins such as CyP-D, HSP10, 60 and 75 were upregulated (Figure 4-7). Upregulation was only observed in HSP70 as demonstrated in Figure (4-6), where a distinctive band is visible at 70 KDA. The Western blot expression of HSP70 is shown in regards to the degree of reperfusion after 1 hour simulated ischaemia. Generally as the reperfusion time increased, the expression of HSP70 increased and reached a peak at 24 hours and declined thereafter. These values are also represented in graphical form (Figure 4-5)

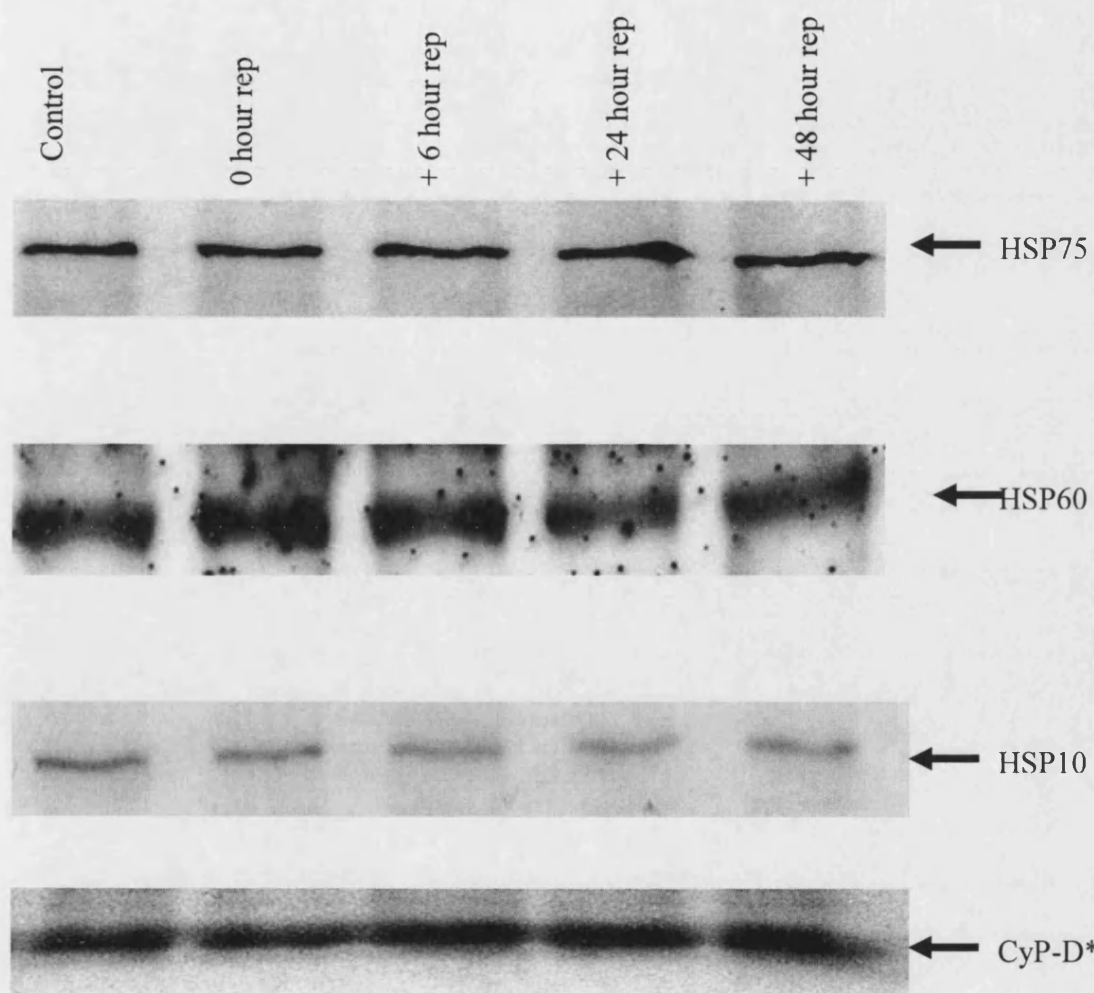


Figure 4-7 Western blot analysis of IPC treated cardiomyocytes

Primary neonatal cardiomyocytes were subjected to 1 hour SI and then reperused for 0, 6, 24 and 48 hours reperfusion. Total proteins were isolated and each sample was electrophoretically separated on a 12.5% SDS/PAGE gel. Following protein transfer each gel was probed with the relevant antibody. *As mentioned in the materials and methods chapter, mitochondria was isolated and the CyP-A antibody was used to probe for CyP-D expression.

4.5 Discussion

Here, an effective protective preconditioning protocol for primary neonatal cardiomyocytes subjected to LSI/R was devised. The analysis of HSP70 Western blot demonstrated that the protein level reached its peak at 24 hours after 1 hr SI and 24 hrs reperfusion. We believe that the protection observed in this study may correlate with the increasing HSP70 levels which have been shown to be protective against ischaemic stress in other studies^{167,169,170} although other factors are also known to be present and are also protective such as activation of p42/44 mitogen-activated protein kinase.¹⁸⁷ Also we cannot exclude the complex protective effects of angiotension II, bradykinin, catecholamines, opioids and endothelin that are released during ischaemia and involved in the early and late stages of preconditioning.¹⁸²⁻¹⁸⁴

Cardiac protection by preconditioning has been well documented and is known to occur as a biphasic phenomenon with an early or “classic” phase developing in minutes and lasting for 2 to 3 hours followed by a “second window of protection” or delayed preconditioning which starts 24 hours after the initial stimulus and lasts for a few days.^{72,104,188} In the model presented here, only the late phase of preconditioning was studied. In studying this phenomenon a simulated ischaemic buffer was used in which myocytes were subjected to a 1 hour ischaemic episode followed by 24 hour reperfusion in normal medium. The SI buffers, which are used in many preconditioning protocols, are glucose free with the addition of sodium lactate to create acidic conditions. Some of the respiratory buffers contain protonophores such as CCCP¹⁸⁹ and respiratory chain inhibitors (NaCN).¹⁴⁶ Sodium dithionite was used here because it is an effective oxygen scavenger and is able to reduce the cytochrome oxidase component of the mitochondrial respiratory chain and these effects are known to be completely reversible.¹⁸⁷

In this study it was found that simulated ischaemia produced delayed protection in a time dependent manner with a maximum effect at 1 hour SI and 24 hours reperfusion. These results correlate with other studies conducted on neonatal cardiomyocytes, whereby metabolic inhibition has resulted in upregulation of HSP70 and increased protection.¹⁶⁹ Other groups have upregulated HSP70 expression by transfection and

found that HSP70 is protective against LSI.^{61,133,145,190} Analysis of protein expression of CyP-A and CyP-D in the cytoplasm and in mitochondria respectively, did not reveal any changes in comparison to their respective controls. Also in the present study no upregulation of mitochondrial chaperones HSP60, 10, and 75 were detected. In this preconditioning model, it is not possible to speculate on the role of these HSP's but, it must be noted that previous studies by other authors have demonstrated in rodent myogenic cell lines and neonatal cardiomyocytes that, overexpression of individual HSP60 and HSP10 does not afford these cells protection against SI.⁶⁹ Only the combined overexpression of these HSP's protects these cells during simulated ischaemia and reperfusion (SI/R) from induced apoptotic cell death.¹⁹¹

Major progress in IPC has occurred over the years since Murry and colleagues⁷² first described the process of preconditioning and Marber and colleagues⁸⁰ defined the "second window of protection" or delayed preconditioning. Although classical preconditioning is more potent, delayed preconditioning is believed to be clinically more practical because of the longer duration of protection not just against myocardial infarction, but also against reperfusion induced myocardial stunning.^{10,104} It is believed that initial triggers of this protective phenomenon are NO, ROS and adenosine. These are transduced by signalling chemicals such as PKC and NF- κ B, and are transported to the nucleus where transcription of iNOS, COX-2 and other cardioprotective genes occurs. In this phase, synthesis of HSP's and activation of K_{ATP} channels occurs.¹⁰⁴ This present study was primarily concerned with the involvement of a limited number of HSP's, (HSP10, 60, 70, 75 and cyclophilin). However, there is growing evidence that other HSP's such as HSP32¹⁹² and HSP27¹⁷⁰ have an important part to play in the sequelae of events involved in IPC. It also appears that certain other HSP's and signalling processes are activated depending on the method of preconditioning.¹⁶⁹

The present study demonstrates that in this ischaemic preconditioning model, upregulation of HSP70 is directly correlated to protection of isolated neonatal cardiomyocytes against LSI/R. The protection observed also preserves $\Delta\psi_m$. It is currently unclear as to whether the mitochondrial chaperonin complex (HSP60, 10 and 75) is upregulated in IPC. Our studies did not reveal any new data regarding this

complex. No difference in enzymatic activity of cyclophilin (PPIase) or protein expression was observed. A lot of work has been carried out on the role of HSP70 in preconditioning; it would be interesting to investigate the degree to which it is involved in IPC. Using compounds such as quercetin to attenuate HSP70 expression¹⁹³ could help to determine its role in IPC in conjunction with other HSP's. Determination of other factors such as oxygen consumption, free radical production and ATP levels could further help to understand the events occurring in this phenomenon. Then it may be possible to develop drugs that can initiate a similar stress response and therefore precondition cells to severe insults. Kume and colleagues¹⁹⁴ used a chemotherapeutic agent, doxorubicin, an anticancer drug, to initiate a stress response within rat liver, which protected against an ischaemic insult. From their results, they believe that pharmacological preconditioning does have a clinical application; however doxorubin has toxicity problems in the heart.¹⁹⁴ Therefore this approach would necessitate the development of novel drugs. Furthermore, although the time course of HSP70 experiments following IPC in our model strongly correlates with the developments of protection, we have not causally linked HSP70 function to the mode of protection. In order to do this, we would have to perform experiments using anti-sense oligonucleotides to HSP70 or an RNA approach. HSP70 knockout *in vivo* is not possible due to its essential role in development of the organism and the heart.

CHAPTER 5

5. HSP EXPRESSION IN SUBSARCOLEMMAL AND INTERFIBRILLAR MITOCHONDRIAL FROM A RAT LAD OCCLUSION MODEL

5.1 Introduction

Mitochondria have been described as “the powerhouse of the cell” as they provide the majority of ATP. Hence loss of mitochondrial function will inevitably lead to cell death. In fact it has been determined that mitochondria not only decide whether a cell will die but also whether cell death is by apoptotic or necrotic pathways.¹⁹⁵ These and other findings have led to a resurgence of interest in mitochondrial physiology during the 1990’s until the present day.

In the process of myocardial ischaemia (specially during periods of ischaemia greater than 30 minutes), damage occurs when the blood flow is re-established, resulting in reperfusion injury, characterised by high Ca^{2+} accumulation, ATP depletion, membrane depolarisation and high phosphate concentrations.^{37,44,49} These factors cause the mitochondria to swell in the process known as mitochondrial permeability transition (MPT) as a result of the formation of the mitochondrial permeability transition pore (MPTP). This is due to the opening of these non-specific pores in the inner mitochondrial membrane, and depending on the duration of pore opening may, result in irreversible damage and ultimately cell death.³⁸

In heart muscle, electron microscopy reveals the existence of two distinct populations of mitochondria by virtue of their different locations,¹⁵⁴ subsarcolemmal and interfibrillar. Subsarcolemmal mitochondria (SSM) reside just below the sarcolemma whereas

interfibrillar mitochondria (IFM) are located between the myofibrils.¹⁹⁶ These different types of mitochondria are affected differently by ischaemia,¹⁹⁷ ageing¹⁹⁸ and cardiomyopathy.¹⁹⁹ SSM and IFM can be isolated by mechanical and enzymatic methods as originally described by Palmer and colleagues¹⁹⁶ and both methods have been reported to produce good, separate yields of both mitochondrial types.¹⁹⁸

Using variations of the isolation procedures first described by Palmer¹⁹⁶ SSM and IFM were isolated. It is known that under normal and pathological conditions, these mitochondrial subsets respond differently in normal and pathological conditions.^{154,197,198,200,201} SSM possess a greater content of the molecular chaperones HSP60 and mitochondrial HSP70 (mHSP70) than IFM.²⁰² During myocardial IR, redistribution of mitochondrial chaperones may occur in SSM and IFM. These are factors that were investigated in the following experiments.

This study aimed to use a rat left anterior descending (LAD) occlusion model to

- Explore the protein expression of HSP's and mitochondrial membrane potential ($\Delta\psi_m$) in the different mitochondrial subsets.
- Investigate enzymatic (PPIase) activity in normal and occluded mitochondrial populations.
- Define ultrastructural differences between the different populations

These aims will help to prove or disprove the hypothesis that;

Myocardial IR will cause $\Delta\psi_m$ to decrease to a greater extent in SSM than IFM due to differences in HSP expression and activity of cyclophilin.

5.2 Methods

5.2.1 Rat LAD Occlusion Model

As previously explained in Chapter 2, hearts from female Sprague Dawley rats, weighing between 250 –300g were subjected to IR and then the hearts were surgically removed and mitochondria isolated. Rats were separated into 6 groups,

Table 5-1. List of groups with their respective LAD occlusion and reperfusion times

Group 1 (control)	Hearts (n=4) surgically removed after 60 minutes with loosely attached suture around LAD coronary artery
Group 2	Hearts (n=4) surgically removed after 60 LAD minutes occlusion as described (section 2.1.5) and perfusion of Evan's blue dye
Group 3	Hearts (n=4) surgically removed after 60 minutes occlusion and 15 minutes reperfusion
Group 4 (control)	Hearts (n=4) surgically removed after 90 minutes with loosely attached suture around LAD coronary artery
Group 5	Hearts (n=4) surgically removed after 90 minutes LAD occlusion
Group 6	Hearts (n=4) surgically removed after 90 minutes occlusion and 15 minutes reperfusion

5.2.2 Electron Microscopy

Following the harvest of SSM and IFM, they were then processed by electron microscopy as previously described in Chapter 2. This was performed in order to

ascertain if there were any morphological differences in SSM and IFM. Also electron microscopy was used as a method of evaluating the purity of the mitochondrial isolations, by assessing the amount of cellular debris relative to single mitochondria in a given field.

5.2.3 *PPIase Activity*

SSM and IFM were isolated from the rat hearts as previously described in Chapter 2. The mitochondria were washed thoroughly and resuspended in HEPES buffer. A Bio-Rad protein assay was performed on the isolated mitochondrial fractions. Then equal amounts of SSM and IFM of the different heart groups were assayed for cyclophilin (PPIase) activity as previously described in Chapter 2.

5.2.4 *Western Blotting*

From the protein measurements, equal amounts of protein were separated on an SDS-12% polyacrylamide gel and after overnight transfer; the membranes were probed with antibodies for HSP10, CyP-D, 60 and mHSP70 (GRP75) for an hour. The appropriate secondary antibodies were added and the blots were developed using the ECL protocol as described in Chapter 2.

5.2.5 *Measurement of $\Delta\psi_m$ in Mitochondria*

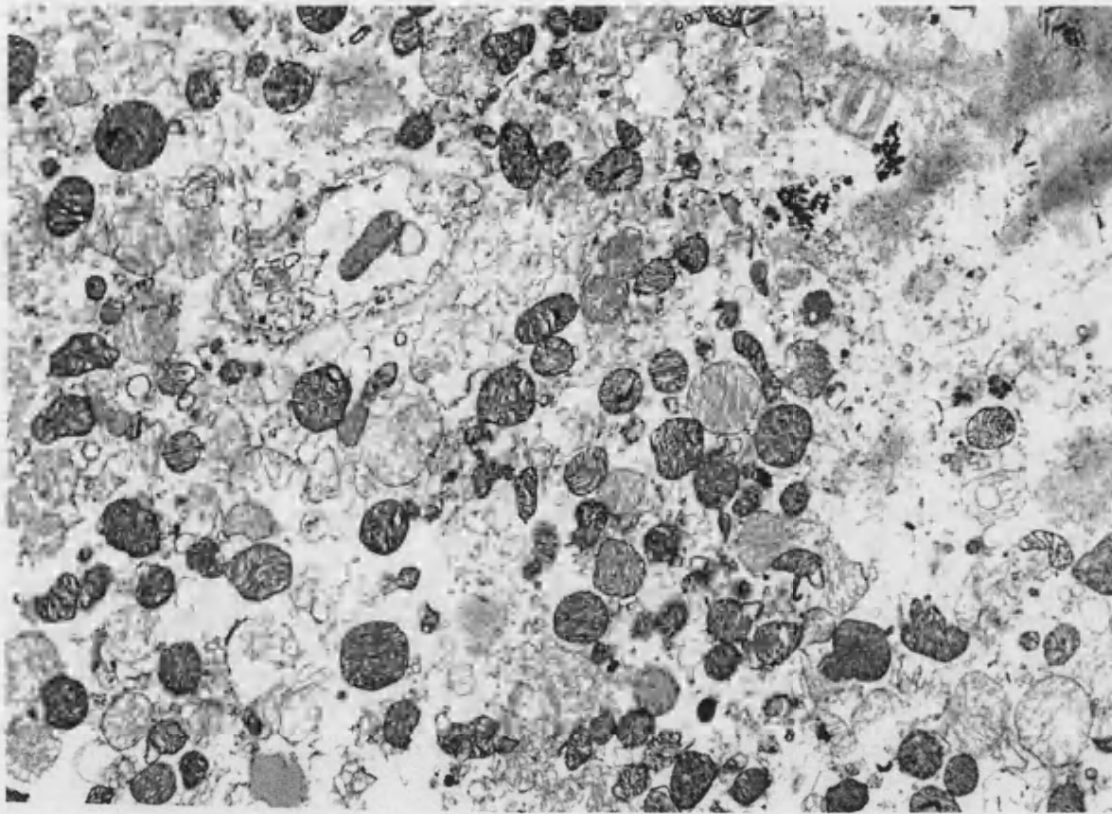
To register the decrease in $\Delta\psi_m$, as previously performed with cardiomyocytes^{150,203,204} a population of rat heart mitochondria were subjected to an uncoupler of oxidative phosphorylation carbonyl *m*-chlorophenylhydrazine (CCCP) that causes a decrease in $\Delta\psi_m$.²⁰³ CCCP is a necessary positive control for $\Delta\psi_m$ as depolarisation of $\Delta\psi_m$ is not always indicative of MPTP formation, but once the MPTP has formed then depolarisation of $\Delta\psi_m$ follows.⁴⁶ The $\Delta\psi_m$ is a component of the proton motive force (pmf) that is generated by the electron transport chain. The formation of the MPTP

results in depolarisation of the $\Delta\psi_m$ as the oxidative phosphorylation that produces ATP and $\Delta\psi_m$ is disrupted.²⁰⁵ Mitochondria isolated from untreated rats were separated into two samples. One sample was treated with 60 μ M CCCP for 30 minutes and the other being the control sample was left untreated. Then both the control and CCCP treated samples were analysed on the fluorescence –activated cell sorter (FACS) after incubation with 0.8nm of the fluorescent probe 3,3'- dihexyloxacarbocyanine iodide DiOC₆ (3). Following this, SSM and IFM from the different groups were incubated with DiOC₆ (3) and any differences between the groups were identified by FACS analysis.

5.3 Results

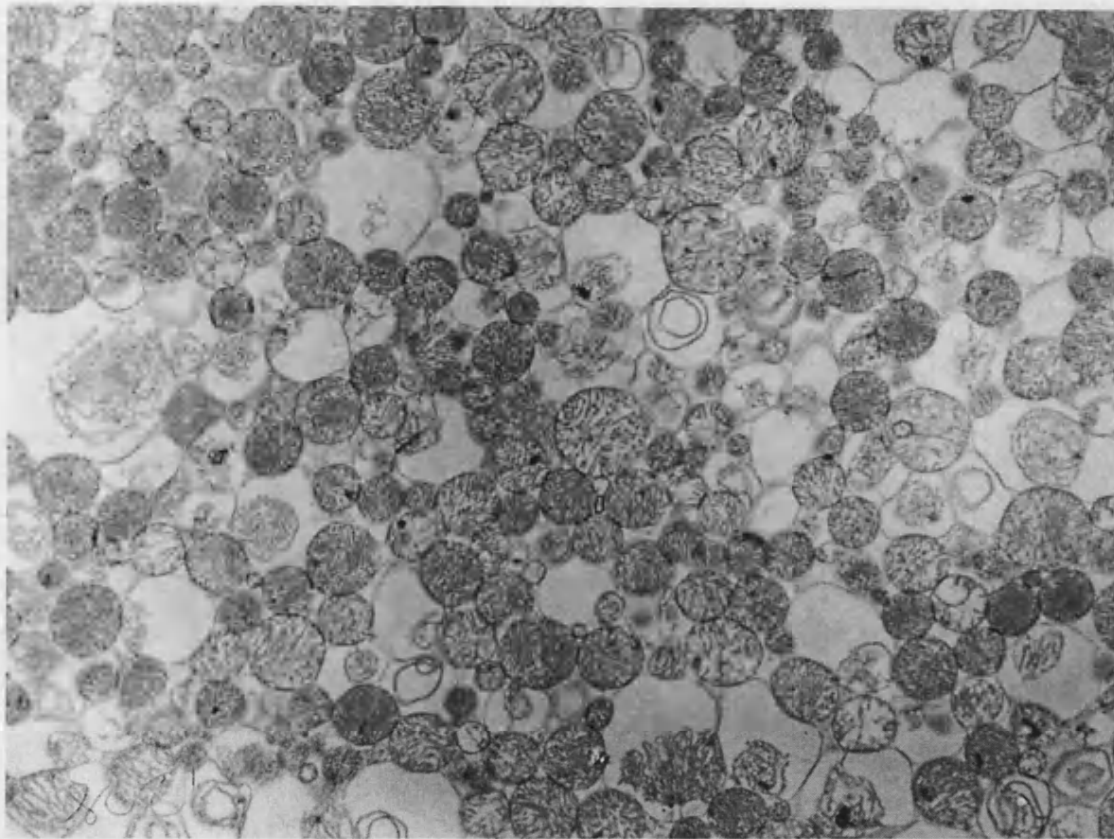
5.3.1 *Electron Microscopy*

Electron micrograph pictures did not reveal any significant ultrastructural differences between the different mitochondria (Micrograph K and L). They do however reveal the purity of the mitochondrial isolations as previously described in Chapter 2, as well as comparing the quantity of mitochondria isolated between the two different populations. Nagarse treatment appears to have released far more mitochondria (Micrograph L) than non-nagarse treated heart and more cellular debris was evident in the SSM population.



Micrograph K. Subsarcolemmal Mitochondria (SSM)

SSM are located below the sarcolemma and are isolated by physical disruption of tissues. Electron microscopy confirms that these mitochondrial populations have maintained their structural integrity after the disruptive isolation process.



Micrograph L Interfibrillar Mitochondria (IFM)

The Figure show non-nagarse treated SSM (Micrograph K) and nagarse treated IFM (Micrograph L) harvests from female Sprague - Dawley control hearts. IFM are located between the myofibrils and they are released by nagarse protease digestion. (Total magnification 10000x)

5.3.2 Changes in $\Delta\psi_m$ in SSM and IFM form the Rat Heart LAD Occlusion Model

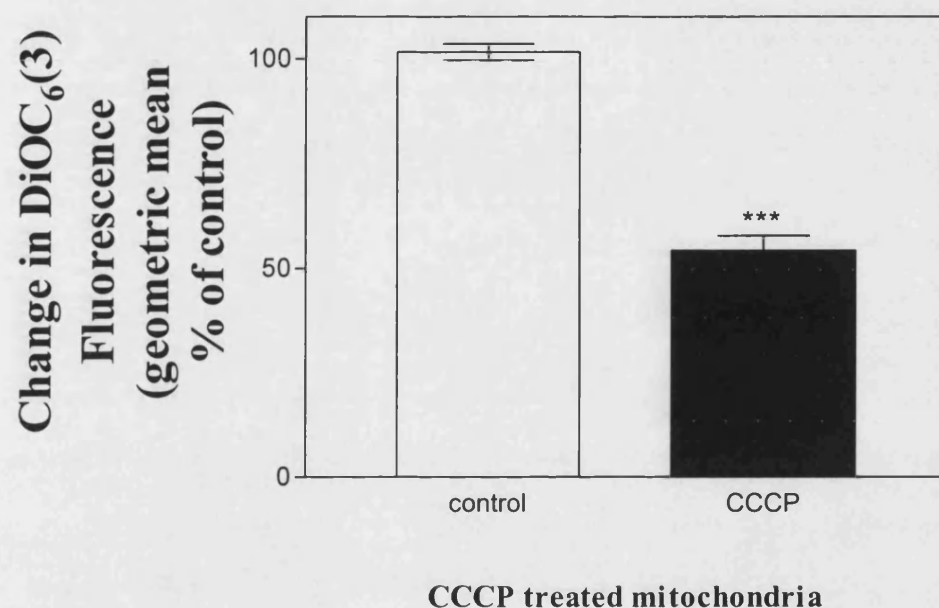


Figure 5-1. CCCP treatment of mitochondria.

This graph demonstrates the change in DiOC₆ (3) fluorescence in mitochondria that have been treated with the protonophore CCCP. Application of CCCP collapses the $\Delta\psi_m$ reducing the fluorescence of the mitochondrial dye DiOC₆ (3) in comparison to the untreated control. CCCP resulted in a significant reduction when compared to the control (**P<0.0001).

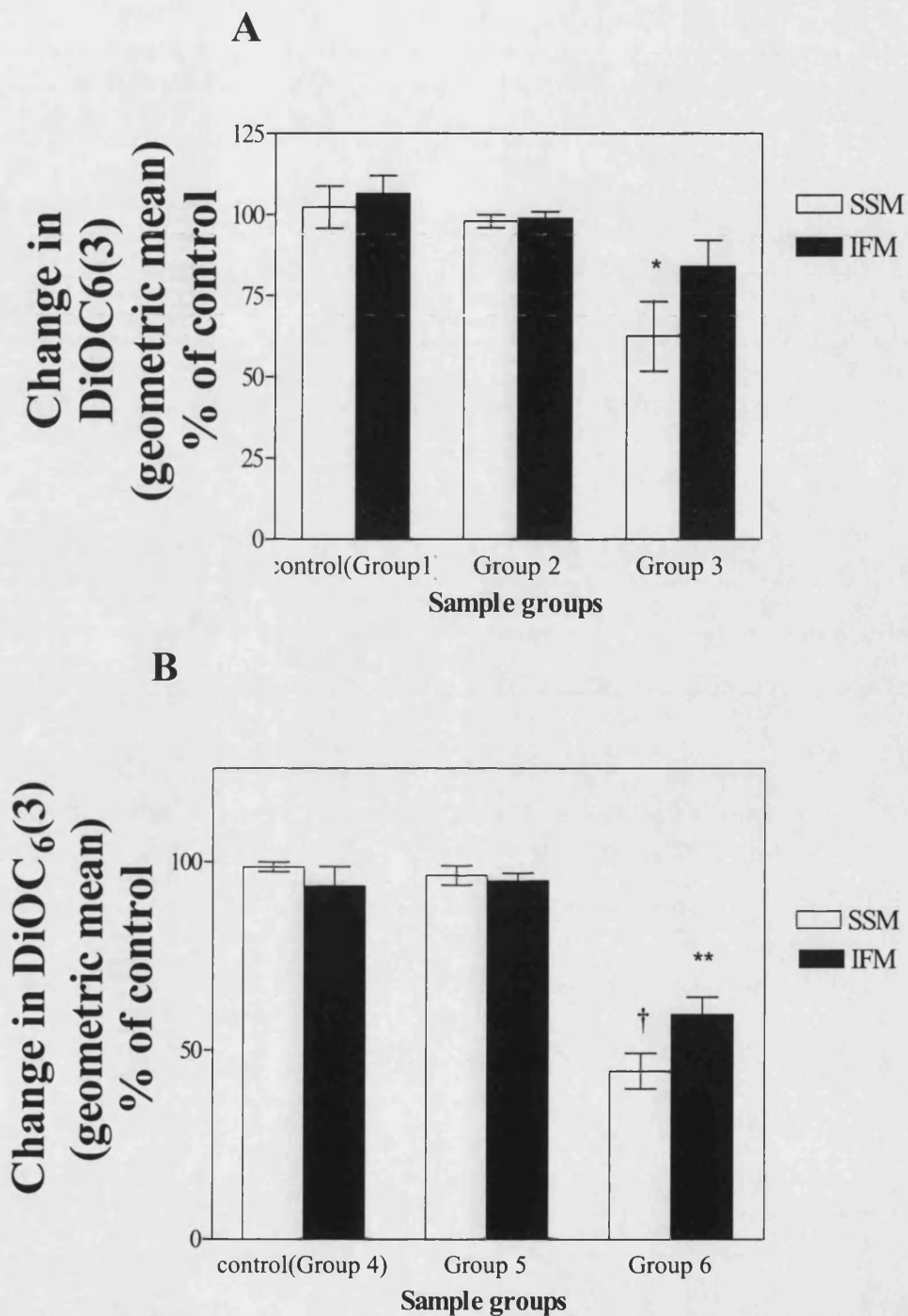


Figure 5-2. SSM and IFM from an LAD occlusion.

SSM and IFM were isolated from a rat LAD occlusion cardiac model. The $\Delta\psi_m$ was measured for all 6 groups and compared as demonstrated in these graphs. In **Figure 5-**

2A, there was no significant difference between the control SSM (Group 1) and Group 2. Group 2 has been LAD occluded for 60 minutes only whereas Group 3 has been LAD occluded for 60 minutes and reperfused for 15 minutes prior to mitochondrial isolation. SSM in Group 3 were significantly different from control (Group 1) (* $P < 0.05$). **Figure 5-2B** is representative of the statistical difference between mitochondria of control (Group 4) and Group 6. Group 6 rats were LAD occluded for 90 minutes and reperfused for 15 minutes only whilst Group 5 rats were LAD occluded for 90 minutes only prior to mitochondrial isolation. † $P < 0.001$ only for SSM when compared to Group 4 (control), ** $P < 0.01$ for IFM (Group 6) in comparison to Group 4 IFM (control). These results are the standard error of the mean of $n=4$ independent experiments.

An uncoupler of mitochondrial oxidative phosphorylation (CCCP) was used, which by attacking the proton gradient across the inner mitochondrial membrane reduces the $\Delta\psi_m^{150}$ and consequently leads to a cessation in ATP production. As in Chapter 3, it was necessary to ensure that we could measure any changes in mitochondrial $\Delta\psi_m$ in the isolated mitochondria from the rat heart LAD model, using the fluorescent probe 3,3'-dihexyloxacarbocyanine iodide (DiOC₆ (3)) on the flow cytometer. In Chapter 3, CsA afforded protection, not statistically significant, but these results were suggestive of an inhibition of the MPTP by an increase in $\Delta\psi_m$ upon pre-incubation of CsA prior to subjection of the cells to LSI/R ion as measured by DiOC₆ (3). Thereby relating $\Delta\psi_m$ to MPTP in these experiments.

On the FACScan, two mitochondrial populations were identifiable as control and CCCP treated. In Figure 5-2A SSM (Group 3) possesses a statistically significantly ($P < 0.05$) decreased $\Delta\psi_m$ as opposed to the SSM control (Group 1). IFM (Group 3) $\Delta\psi_m$ decreased but not statistically different in respect to control IFM (Group 1). Group 2 mitochondria from 60 minute LAD occluded rats demonstrated no change in respect to control SSM/IFM in Group 1.

Figure 5-2B demonstrates the significant reduction in $\Delta\psi_m$ of both SSM and IFM (Group 6) (** $P < 0.001$) as opposed to $\Delta\psi_m$ of SSM and IFM of their respective controls (Group 4). Group 6 rats were LAD occluded for 90 minutes and reperfused for 15 minutes only prior to mitochondrial isolation whereas Group 5 rats were LAD occluded

for 90 minutes only. There was very little change between Group 5 rats and control (Group 4) occlusion with 15 minute reperfusion.

5.3.3 Heat Shock Protein Expression in SSM and IFM

There were no observed significant differences in expression of heat shock proteins in normal, ischaemic or reperfused SSM and IFM. We compared HSP10, 60, mHSP70 and CyP D expression. (Figure 5-3).

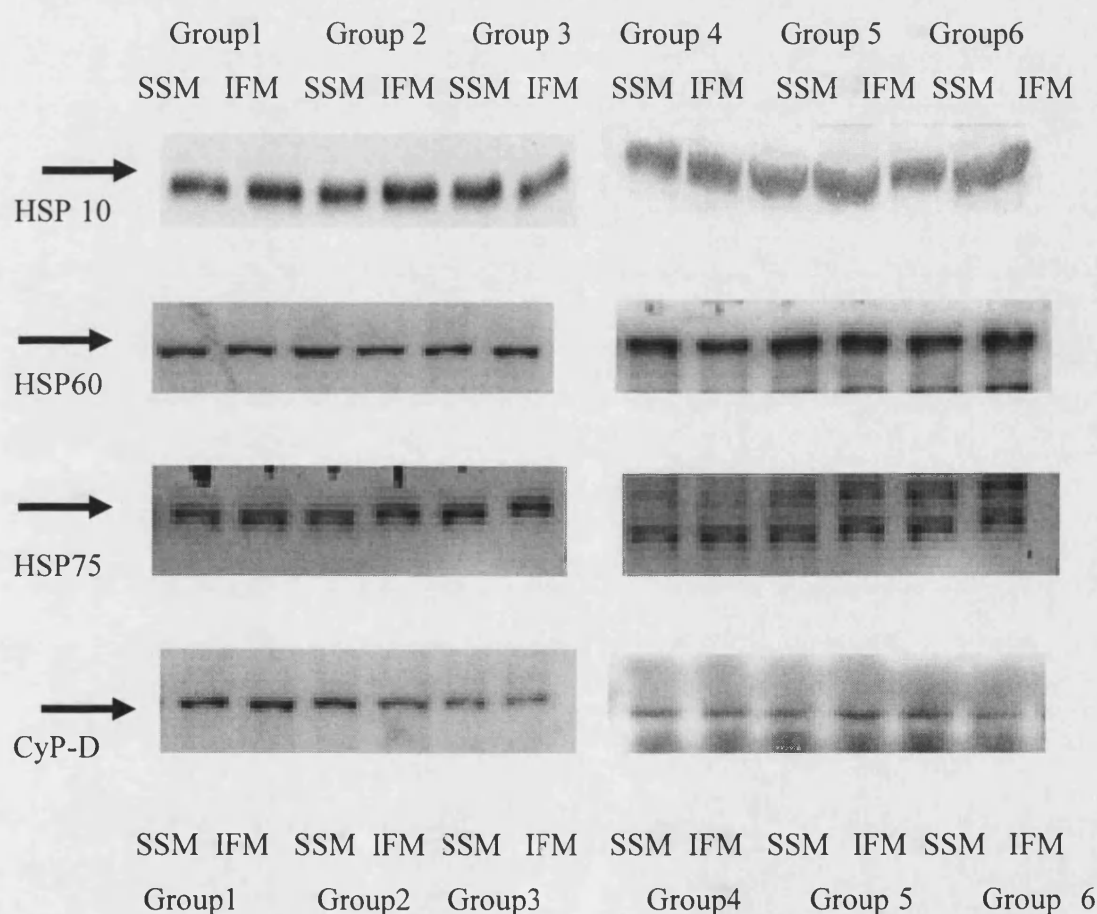


Figure 5-3 Western blot analysis

Western blot showing protein expression of rat heart cardiac mitochondrial fractions, after incubation with a panel of antibodies HSP10, HSP60, HSP75 and CyP-D. There is no change between SSM and IFM before or after ischaemia and reperfusion.

5.3.4 PPIase Activity in SSM and IFM

The enzymatic activity of cyclophilin was measured between SSM and IFM in normal heart tissue as well as in ischaemic and reperfused. The activity of cyclophilin-D (CyP-D) was assessed, which as already mentioned is an integral member of the MPTP that plays a role in cell death. No difference in PPIase activity between SSM and IFM in normal heart tissue was observed. The same observation was seen in PPIase activity between SSM and IFM in the ischaemic and reperfused groups. Even between SSM and IFM between normal, ischaemic and reperfused the same results were obtained irrespective of the degree of reperfusion.

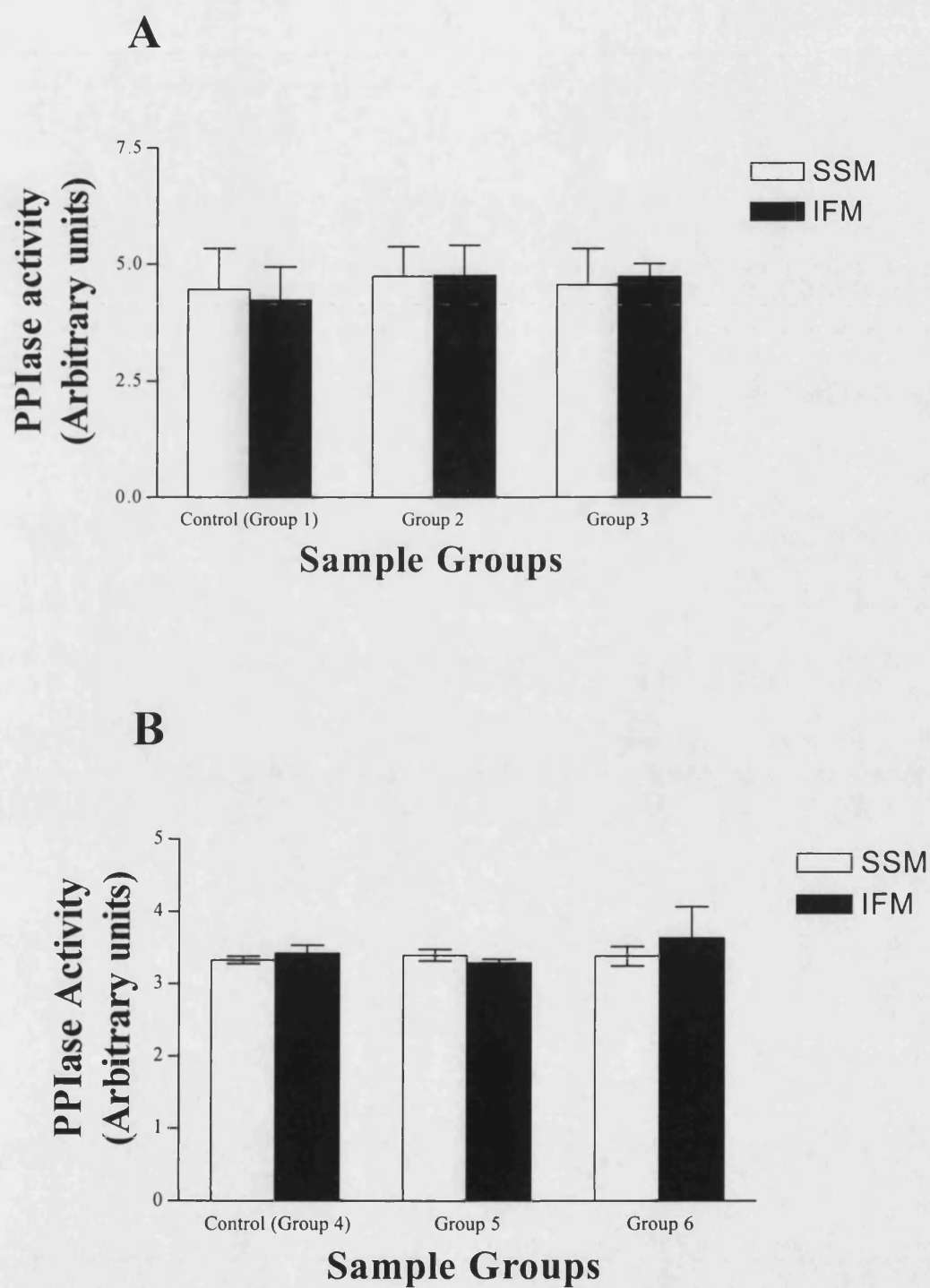


Figure 5-4. PPase activity of mitochondria isolated from LAD occluded rats

SSM and IFM were isolated from rats, which have been occluded and reperfused for differing time periods (as described in the materials and methods). There was no

statistical difference in PPIase activity between any of the groups and their respective controls as visualised in **Figure 5-4A** and **5-4B**.

5.4 Discussion

The objectives were to emulate myocardial ischaemia/reperfusion using a rat heart LAD occlusion model, in order to examine the effect on:

- The $\Delta\psi_m$ in the different groups in comparison to their respective controls
- Heat shock protein and cyclophilin expression and activity in SSM and IFM
- Any ultrastructural differences.

In order to achieve these objectives, hearts from female Sprague Dawley rats were used and organized into 6 groups. Groups 1 and 4 were the controls for 60 and 90 minutes ischaemia only (Groups 2 and 5), and groups 3 and 6 were subjected to 60 and 90 minutes ischaemia with 15 minutes reperfusion. The method used for isolating SSM and IFM were adaptations of established protocols,¹⁹⁶ and electron microscopy helped to confirm purity of isolated samples as previously described.

The main findings in this study are that in this model, $\Delta\psi_m$ decreased significantly when compared to their respective controls only in the reperfusion groups (3 & 6) in SSM ($p<0.05$) ($p<0.001$) respectively. In IFM the decrease was significant only in group 6 ($p<0.01$). Our findings are partly consistent with our initial hypothesis, SSM sustain increased damage during IR in comparison to IFM, possibly due to their close proximity to the plasma membrane. Ultrastructural analysis did not reveal any major differences between these different types of mitochondria, which correlates with what has been observed by other researchers.^{154,198,206} There were no differences in the PPIase assay between the control and treated samples, and the same was found for expression of the heat shock proteins (HSP10, HSP60, mHSP70 and CyP-D) tested.

Our findings are not wholly in agreement with previously published data. Gatellitelli and colleagues²⁰⁷ in measuring total mitochondrial Ca^{2+} discovered that Ca^{2+}

accumulated to a far greater extent in SSM than IFM in guinea-pig cardiac myocytes when they are loaded with calcium and sodium and potentiated, whereas in resting cells the levels of Ca^{2+} were equal. This may help to explain why in experiments upon subjection to ischaemia /reperfusion, $\Delta\psi_m$ decreased to a greater degree in SSM than in IFM. It is a known fact that one of the major contributors to ischaemic cell death is high concentrations of cytosolic Ca^{2+} .²⁰⁸⁻²¹⁰ Subjecting these myocytes to ischaemia and reperfusion may alter the levels of Ca^{2+} in SSM. In many cells there is a continuous shuttling of Ca^{2+} between the mitochondria and endoplasmic reticulum. Disruption of this movement results in overloading of Ca^{2+} in the mitochondria. This activates processes leading to the formation of the MPTP and therefore cell death ensues.^{37,210,211} As a consequence of the formation of MPTP, oxidative phosphorylation that maintains $\Delta\psi_m$ is disrupted and therefore there is a decrease in $\Delta\psi_m$.⁴⁶

In the study reported in this Chapter, it has been demonstrated that in this model of LAD occlusion, there was no difference in expression of mitochondrial heat shock proteins in ischaemically reperfused compared with normal SSM and IFM. In other studies mitochondrial chaperone expression has been found to be absent in SSM from muscle suffering from respiratory chain deficiency,²¹² but overexpressed in the corresponding IFM. This would indicate that there was also a reduction or absence of protein import in SSM. Previous work has established the importance of HSP60 for cell survival²¹³ and refolding²¹⁴ as well as the importance of mHSP70 in its role as an ATP dependent import “motor”, importing precursors into the mitochondrial matrix. Takahashi and colleagues²⁰² upon investigating protein import into mitochondria discovered that both HSP60 and mHSP70 levels are present in the mitochondrial subpopulations but their levels are increased in SSM in ATP depleted rat muscle, although the rate of protein import is greater in IFM. They suggested this contradiction was probably due to the rate of mitochondrial import, which is determined by ATP consumption, which is greater in IFM.

Battersby and colleagues²⁰⁶ isolated mitochondrial subpopulations from below the cell membrane and in the myofibrils, using classical methods of isolating these subpopulations¹⁹⁶ in rainbow trout. They were not able to discern any biochemical or

genetic differences between the two populations. They speculated that although these populations appear to be identical, they might be regulated differently *in vivo*, as their location could also provide differential rates of access to mitochondrial substrates. This contrasts with mammalian muscle, which possesses biochemically distinct populations.^{196,197,207} Although Battersby and colleagues looked at some respiration rates, they did not examine levels of mitochondrial chaperones within these subpopulations. Import and processing of mitochondrial peptides do contribute to mitochondrial heterogeneity^{202,215} Other investigators have looked at the effect of aging on these subsets^{154,197,198,200} and have discovered that oxidative metabolism and protein yield declines with age in IFM in a rat model of aging, but SSM are unaffected by these aging related decreases.

In summary, this present study has demonstrated that ischaemia/reperfusion damage decreases mitochondrial $\Delta\psi_m$ SSM to a greater extent than IFM in the LAD rat occlusion model. It is likely that SSM, because of their locality, are more susceptible than IFM to damage from external stresses. Also SSM, as in the potentiation experiments of Galitelli¹⁷¹ were found to contain double the amount of calcium as opposed to IFM, which may mean that SSM possess a greater propensity to form MPTP, as Ca^{2+} is one of the major inducer of pore formation⁸⁶. This may help to explain the results of $\Delta\psi_m$ experiments, but further experiments involving sodium and calcium loading in ischaemic tissue need to be conducted in correlation with $\Delta\psi_m$. It has been suggested that the protein import pathway may be dependent on $\Delta\psi_m$ ²⁰². As in these experiments SSM demonstrated a lower $\Delta\psi_m$ than IFM, it would be interesting to ascertain if conditions which decrease $\Delta\psi_m$ cause the rate of protein import to decrease also. $\Delta\psi_m$ decreases in protein import would decrease in parallel as ATP is necessary to maintain proton motive force (pmf) (which in turn sustains $\Delta\psi_m$.) and protein import. Preservation of mitochondrial function is very important to the cell; therefore further experiments involving a global preconditioning protocol could be used and then determine whether protection was afforded to one or both of the mitochondrial subsets.

CHAPTER 6

6. THE ROLE OF NITRIC OXIDE IN PROTECTION OF THE MYOCARDIUM AGAINST OXIDATIVE STRESS

Data from this chapter has recently been published Ekaterina Monastyrskaya. Najeem Folarin, Igor Malyshev, Colin Green and Larisa Andreeva: Application of the nitric oxide donor SNAP to cardiomyocytes in culture provides protection against oxidative stress. Nitric Oxide.2002 Sep; 7(2): 127.

6.1 Introduction

Furchgott, in 1989 ¹²⁵ suggested that endothelial derived relaxing factor (EDRF) and nitric oxide (NO) were one and the same. Ever since then, NO has been considered to be a very important molecule, such that in 1993 it was voted "molecule of the year" due to its role as a universal regulator of physiological and defence mechanisms. ²¹⁶ Some of its roles include mediation of the cytotoxic immune response, regulation of vascular tone in the cardiovascular system as well as neurotransmission in the nervous system ¹⁸². Its absence or insufficiency is thought to be a contributory factor in the pathogenesis of vascular diseases such as hypertension, atherosclerosis and myocardial ischaemia. ²¹⁷

NO is generated from the oxidation of the substrate amino acid L-arginine and an enzyme, NO synthase (NOS) of which there are several known isoforms including neuronal NOS (nNOS), endothelial or constitutive NOS (ecNOS) and inducible NOS (iNOS) ¹²⁴. NO is a labile compound, which reacts spontaneously with molecular oxygen to produce numerous oxides, including nitrogen dioxide (NO₂), dinitrogen

trioxide (N_2O_3) and nitrite (NO_2^-).¹²⁴ Some of these oxides will nitrosate primary and secondary amines producing potentially carcinogenic nitrosamines.¹²⁴ NO reacts with reactive oxygen species such as hydrogen peroxide (H_2O_2) and its reaction with the superoxide ion (O_2^-) yields a potent oxidant and cytotoxic agent the peroxynitrite anion (ONOO^-).¹²¹

There are many scientific reports about NO functions. In macrophages, NO is released in large quantities as part of a cytotoxic defence mechanism.¹²⁴ Bolli and colleagues have demonstrated that iNOS is involved in delayed preconditioning.^{100,218} Some investigators have reported on the cytoprotective properties of NO donors afforded to cardiomyocytes undergoing reperfusion injury⁴⁸ whilst others have observed that peroxynitrite damages vascular endothelium.⁴⁴

Evidence is accumulating to support the role of NO and peroxynitrite in modulation of mitochondrial functions.^{51,121} NO can reversibly inhibit cytochrome oxidase whereas peroxynitrite irreversibly inhibits it, causing mitochondrial dysfunction. Addition of peroxynitrite to mitochondria results in the opening of a non-selective channel for solutes of $<1.5\text{kDA}$ ¹²¹ called the mitochondrial permeability transition pore (MPTP),¹¹⁶ regulated by Ca^{2+} concentrations, ROS and depleted adenine nucleotides.²⁵ The formation of the MPTP causes respiratory inhibition²⁵ and may result in the release of apoptogenic factors from the mitochondria thereby resulting in apoptosis²¹⁹ and ultimately necrosis. Furthermore it has been demonstrated, that NO and peroxynitrite can control Ca^{2+} efflux which determines whether or not the MPTP forms with the concomitant collapse of $\Delta\psi_m$.¹²⁸

It has been suggested that NO participates in many complex signalling pathways, such as activating heat shock proteins. Malyshev and colleagues²²⁰ have reported that NO can induce the synthesis of HSP70. We have observed that NO activates cyclophilin and HSP70 in cardiomyocytes (abstract from cold spring harbour meeting). We propose that: 1) NO induced cyclophilin and HSP70 contribute to protection against IR injury; 2) NO donors confer protection against oxidative stress by modulating $\Delta\psi_m$.

The aim was to subject cells to oxidative stress after pre-treatment with the nitric oxide donor S nitroso N acetylpenicillamine (SNAP) in order to access the effect on:

- Changes in mitochondrial membrane potential
- Cell viability
- Expression of CyP-D, HSP70, HSP75 and HSP60 (mitochondrial chaperone complex)

6.2 Methods

6.2.1 Cell Culture

H9c2 cells were obtained from the American Type Culture Collection (CRL-1446, ATCC Rockville, MD, USA). H9c2s were grown until 50% confluence in culture vessels and incubated with S nitroso N acetylpenicillamine (SNAP) which was at a final concentration of 1mM in full growth medium (FGM) consisting of Dulbecco's Modified Eagle Medium (DMEM) with sodium pyruvate and 1000mg/l glucose (Gibco-BRL) and 10% foetal calf serum in 95% atmosphere and 5% CO₂. Cells were seeded into 6 well culture dishes for viability assays or left in 75cm² flasks a day before experiments were conducted. The nitric oxide donor SNAP (Sigma) was initially dissolved in DMSO to create a 1M stock solution and kept at –20°C until used.

6.2.2 Cell Viability Assays

MTT assays were performed as previously described in Chapter 2.

6.2.3 Trypan Blue Exclusion Assay

Treated cells were incubated with a 0.8% Trypan blue solution (Sigma) and then using a haemocytometer the average number of viable cells able to exclude this dye was calculated.

6.2.4 Mitochondrial Membrane Potential ($\Delta\psi_m$) Assay

This assay was performed as previously described in Chapter 2

6.3 Results

In order to evaluate the effect of oxidative stress and NO on the onset of the MPT we assessed the $\Delta\psi_m$ by incubating the cells with DiOC₆ (3) a lipophilic cationic dye.²²¹

Various concentrations of the NO donor SNAP were incubated with H9c2 cells for 2 hours, then reperused for 24 hours and harvested for cell viability analysis. This was performed in order to ascertain the optimum concentration to be used in these experiments (Figure 6-1), which was decided to be 1mM.

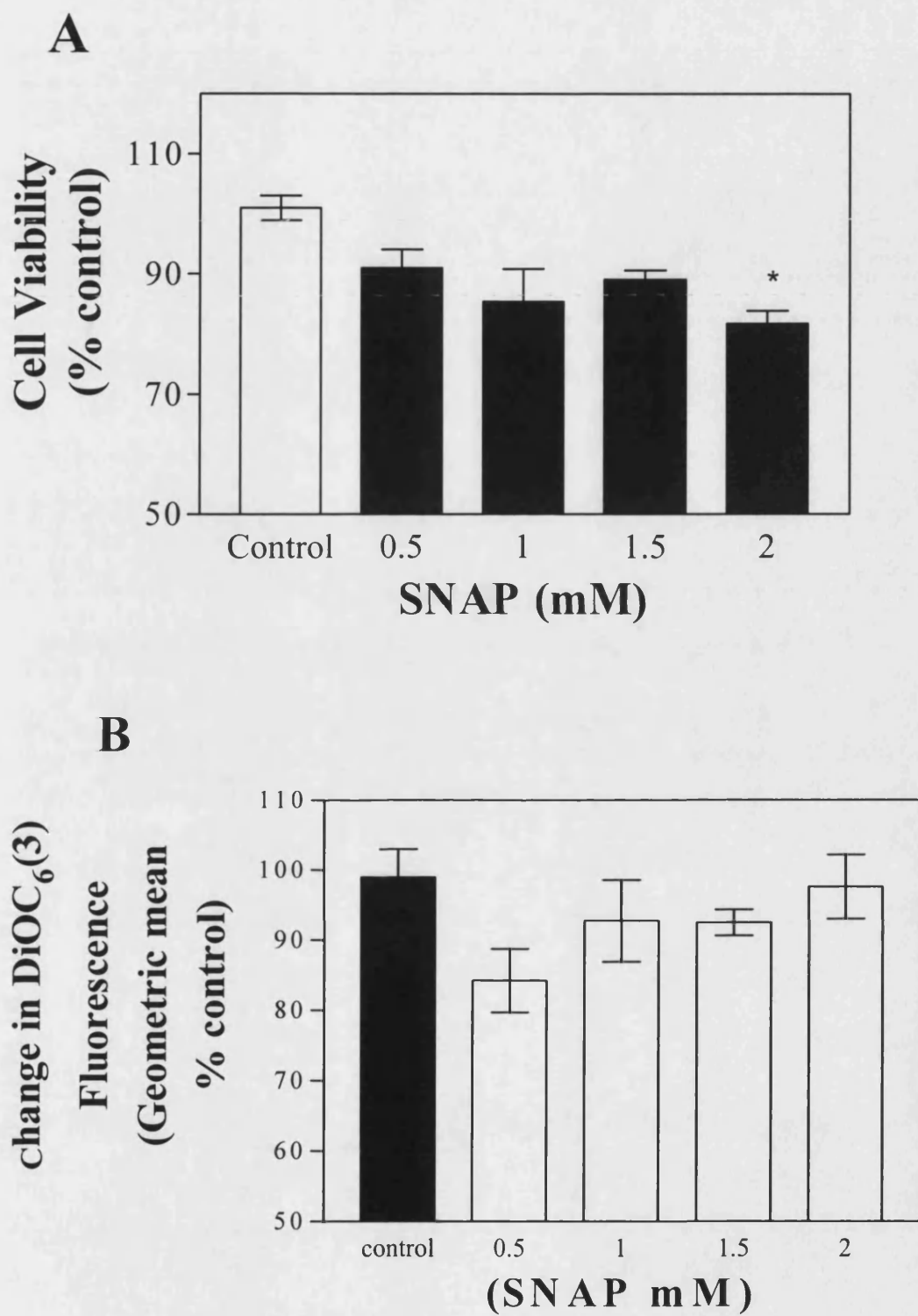


Figure 6-1. Viability of H9c2 cells treated with various concentrations of SNAP.

Cells were incubated with SNAP for 2 hours, washed and reperused for 24 hours prior to analysis for **(A)** MTT cell viability assay and **(B)** change in DiOC₆ (3) fluorescence as a measure of the mitochondrial membrane potential ($\Delta\psi_m$). In graph **(A)**, only 2mM SNAP exhibits statistically a significant decrease in viability (*P<0.05} in comparison to the control. There is no statistically significant decrease in DiOC₆ (3) ($\Delta\psi_m$) **(A)** for any of the SNAP concentrations. Data is expressed as S.E.M for 3 pooled separate experiments (n=9). *P<0.05 represents statistical significance in comparison to the control

Pre-treatment with 1mM SNAP elicited a definite resistance to 50 μ M H₂O₂ in H9c2 cells. Those cells that had not been pretreated with SNAP showed a significantly marked reduction in $\Delta\psi_m$ (Figure 6-2) when compared to untreated control cells.

Cells not reperused prior to challenge with oxidative stress show a 10% difference in $\Delta\psi_m$ Figure 6-2A when compared to Figure 6-2B.

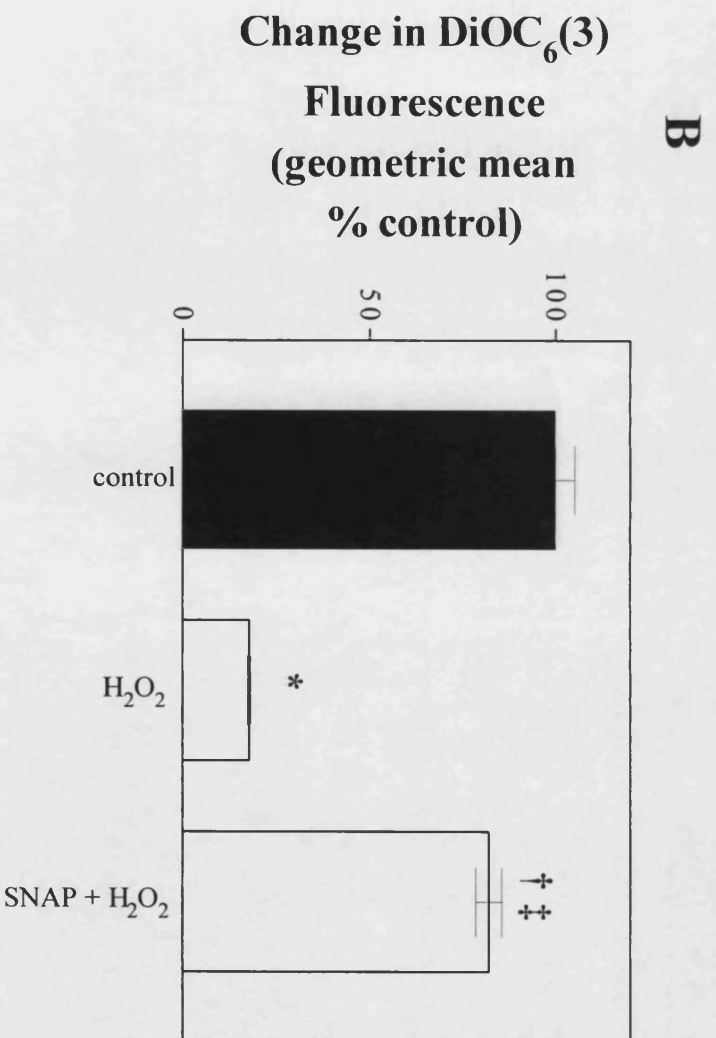
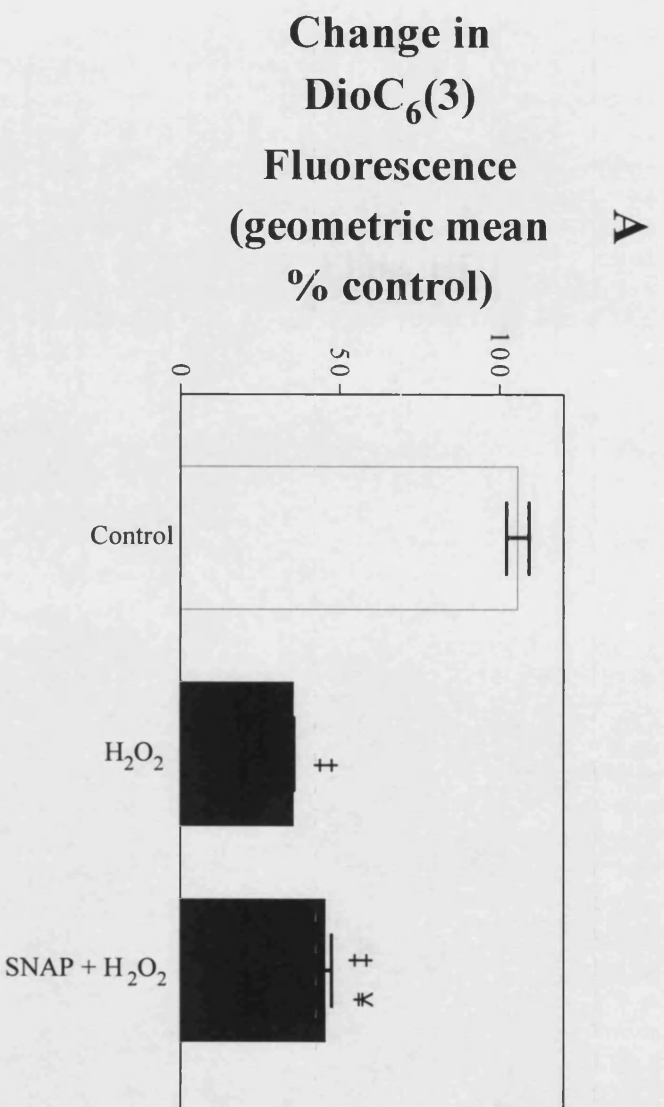


Figure 6-2 Change in DiOC₆ fluorescence after SNAP treated H9c2 were exposed to 50 μ M H₂O₂.

The $\Delta\psi_m$ was monitored by the change in DiOC₆ (3) fluorescence after H9c2 cardiomyocytes were treated with SNAP and H₂O₂ as described in the materials and method. After 2 hour pre-incubation with 1mM SNAP, H9c2 cells were reperused for 0 hours (A) and 24 hour (B) prior to 1 hour exposure to 50 μ M H₂O₂ and the fluorescence changes in DiOC₆ (3) were determined by FACS analysis. H9c2 display a significantly increased preservation of $\Delta\psi_m$ in (B) than comparison to (A). Data is representative of the S.E.M of n=9 from 3 pooled experiments. *P<0.001 v control, ‡P<0.05 v H₂O₂. †P<0.001 v H₂O₂; ‡P<0.01 v control

This difference rises to a maximum in those cells that have been reperused for 24 hours prior to exposure to oxidative stress after SNAP incubation; this difference is 60% (Figure 6-3A) when H₂O₂ and H₂O₂ + SNAP treated cells are compared. The correlation between $\Delta\psi_m$ and cell viability is demonstrable by Figure 6-2A, 6-2B, 6-3A & 6-3B where cells which have been pretreated with SNAP demonstrate a higher cell survival rate in contrast to those which have not had this pretreatment. Although the resistance afforded to H9c2 cells was not as elevated in Figure 6-3A, as observed in Figure 6-3B, the difference between H₂O₂ only and H₂O₂ + 1mM SNAP was significant (‡P<0.05).

In the MTT assay (Figure 6-3A and B), the relative protective response of SNAP treated cells is visually different between these two. This response parallels the protective response to $\Delta\psi_m$ decrease as observed in Figures 6-2A and 6-2B following 0 and 24 hours reperfusion prior to exposure to H₂O₂ mediated oxidative stress. Demonstrating the greater protection after 24 hours reperfusion, as opposed to 0 hours reperfusion and subsequent exposure to oxidative stress. Figure 6-3B demonstrates that a 2 hour incubation and subsequent 24 hour reperfusion time course with 1mM SNAP and the vehicle (DMSO) has no toxic effects on H9c2 cells over the time course of these experiments.

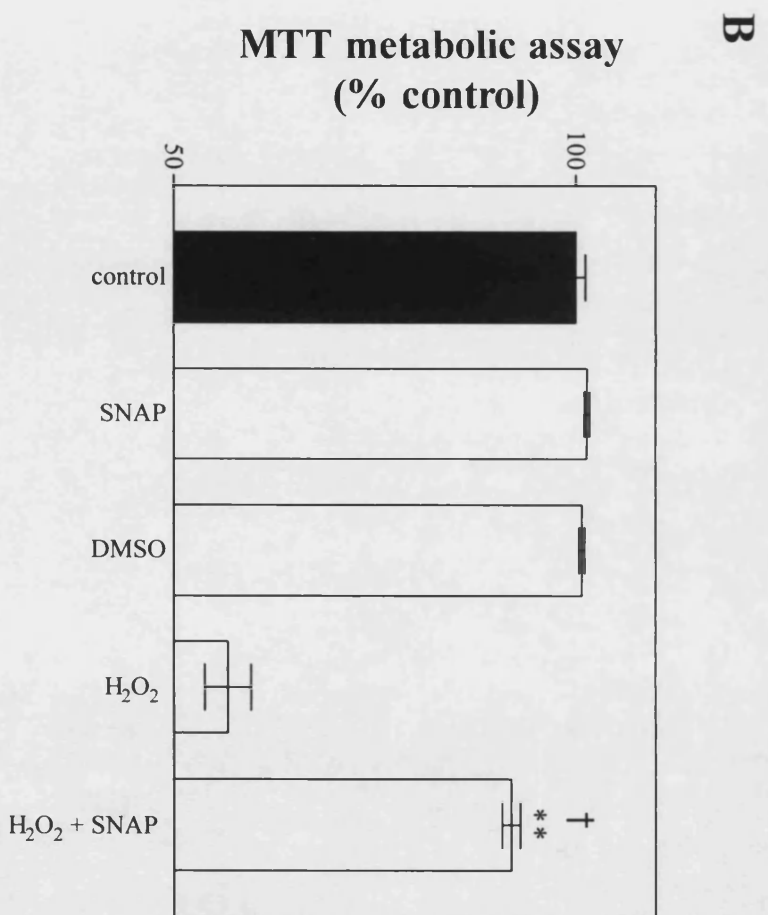
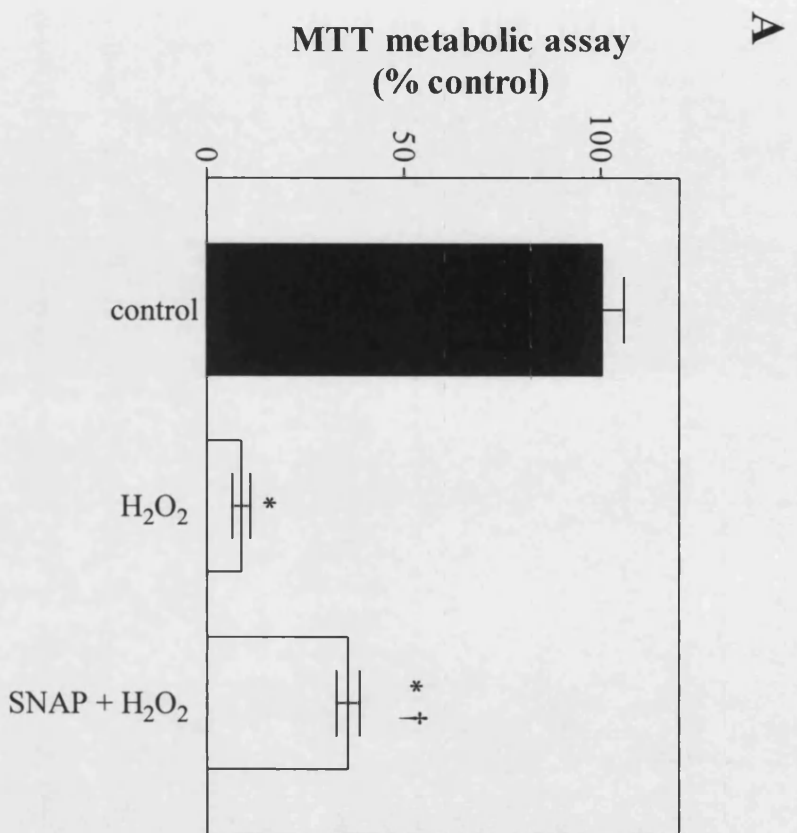


Figure 6-3 Viability of cardiomyocytes after exposure to H₂O₂ and SNAP.

SNAP afforded significant protection of H9c2 cells against 1 hour exposure to 50 μ M H₂O₂ after 0 hours **(A)** and 24 hours **(B)** reperfusion as determined by MTT analysis. Graph **(Figure 6-3B)** demonstrates that there are no damaging effects from incubating these cardiomyocytes with DMSO and SNAP for 2 hours with 24 hours reperfusion. These results represent means \pm S.E.M of 6 separate experiments for each group (n=6). **(Figure 6-3A)** *P<0.001 v control; †P<0.001 v H₂O₂. **(Figure 6-3B)** †P<0.001 v H₂O₂ , **P<0.05 v control.

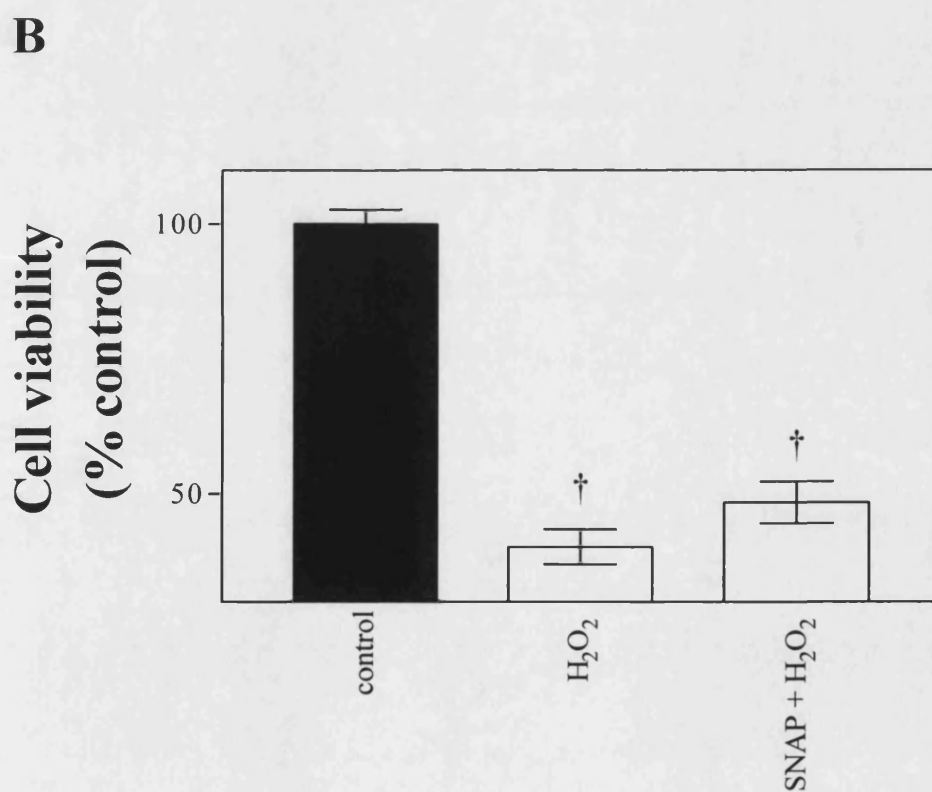
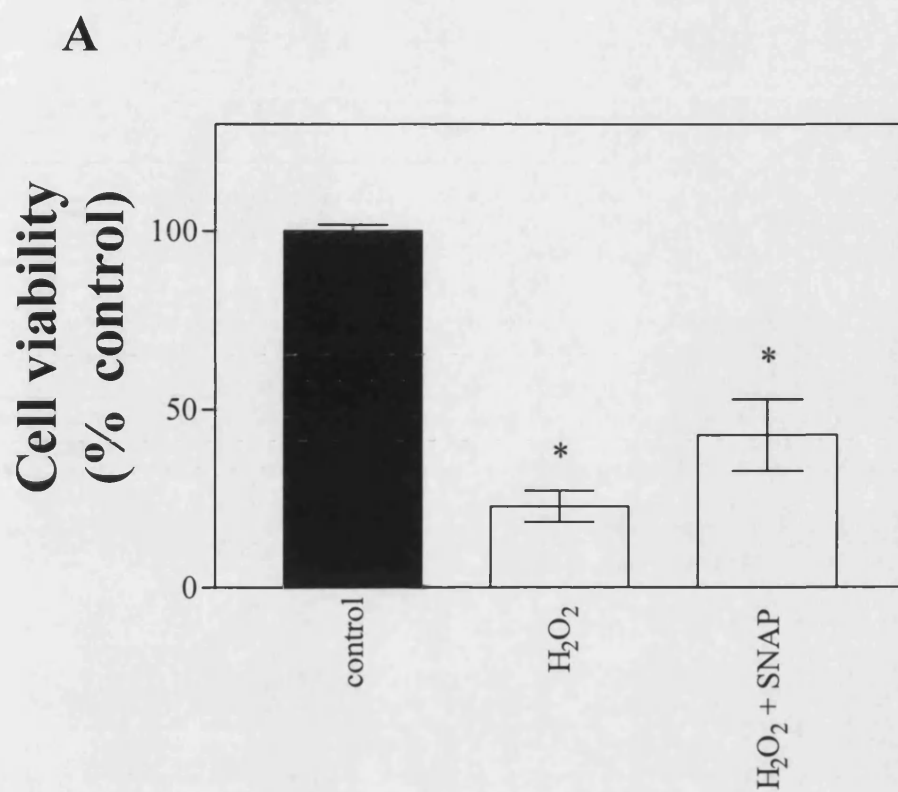


Figure 6-4. Survival of SNAP treated H9c2 cells after exposure to H₂O₂

After subjection to 1 hour 50 μ M H₂O₂ without (**Figure 6-4A**) and with (**Figure 6-4B**) 24 hour reperfusion as previously described in the materials and method. Cells were assayed for viability by trypan blue. The survival rate of cells treated with H₂O₂ was also recorded and plotted. However, protection afforded to these cardiomyocytes was not statistically significant without (**Figure 6-4A**) and with (**Figure 6-4B**) 24 hour reperfusion prior to exposure to oxidative stress. Data are means S.E.M of 6 independent experiments. *P<0.01 vs control; †P<0.001 vs control

As a sign of irreversible damage to cardiomyocytes caused by loss of plasma membrane integrity, cardiomyocytes able to exclude the blue dye were recorded as part of the trypan blue assay as shown in Figure 6-4A and 6-4B. There is virtually no difference between the two graphs in survival rates after H₂O₂ exposure is virtually the same immediately after SNAP incubation (Figure 6-4A) after 24 hour incubation prior to subjection to oxidative stress (Figure 6-4B).

6.3.1 Mitochondrial Heat Shock Protein Expression in Cells Exposed to Oxidative Stress and Nitric Oxide donor SNAP

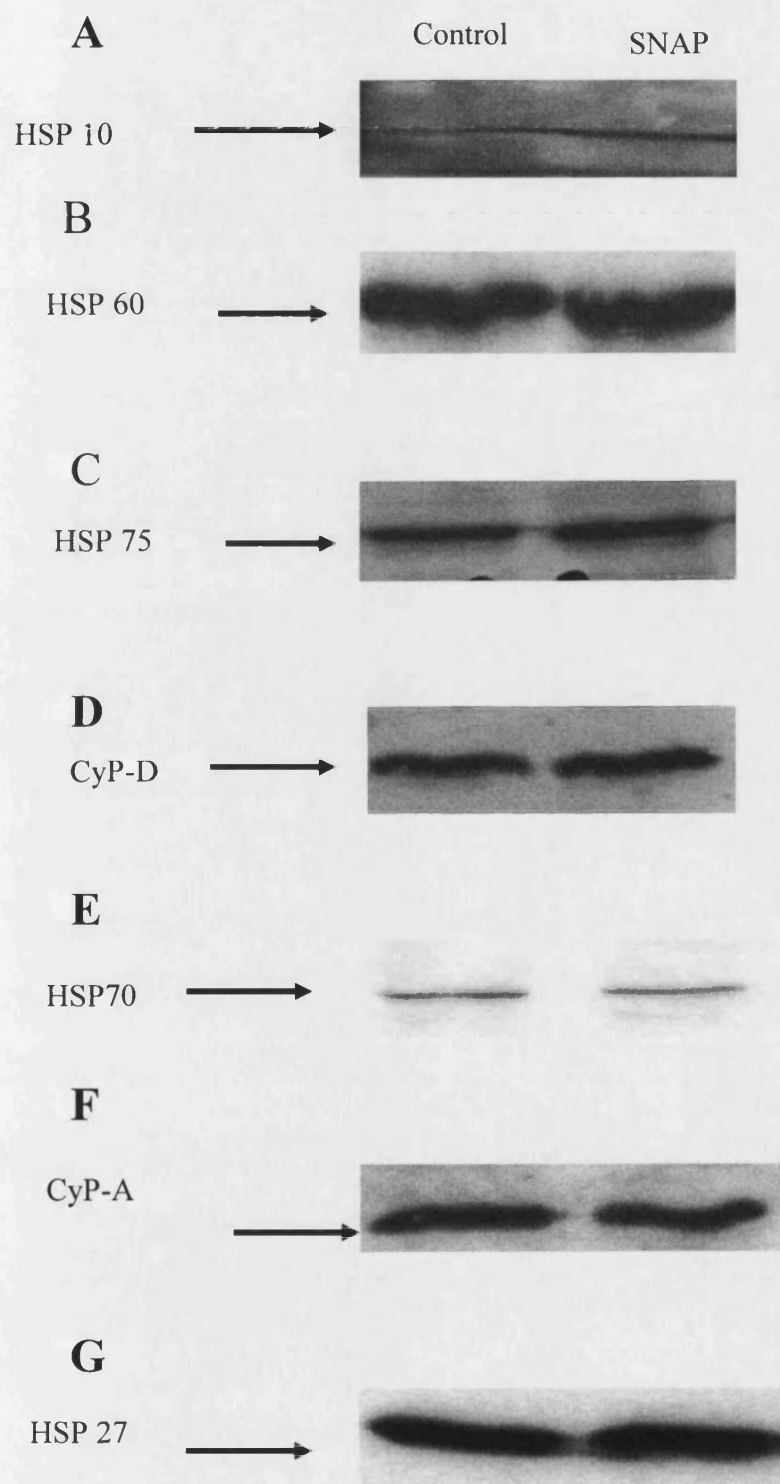


Figure 6-5. Western blot expression of SNAP treated cells.

Cardiomyocytes were left untreated (control), subjected to 2 hour incubation with 1mM SNAP only, reperfused for 24 hours before they were harvested and probed with antibodies recognising (A) HSP10, (B) HSP 60, (C) HSP 75 (E) CyP- D, (F) HSP70, (G) CyP-A and (H) HSP27 epitopes. In order to analyse the CyP-D expression, mitochondria were isolated from the treated samples as previously described in Chapter 2. Protein was extracted from these mitochondrial isolations and equal quantities of protein were run on a SDS-acrylamide gel and probed for mitochondrial HSP60 (**Figure 6-5D**) in order to confirm purity of the mitochondrial isolations. Then the antibody for cyclophilin A was used to probe these mitochondrial samples and any expression detectable around 20kD would be due to CyP-D (**Figure 6-5E**) as this is the only cyclophilin present in mitochondria.

As is demonstrated in Figure 6-5, there is no significant induction of any heat shock protein apart from mitochondrial HSP75 and a slight induction of HSP27. All these experiments were repeated 3 times and HSP75 and HSP27 expression levels were elevated in all. Following densitometry it was only HSP75, whose levels were elevated significantly.

6.4 Discussion

Nitric oxide is known to be a major molecule in the regulation of various physiological processes in the immune, nervous and cardiovascular systems. It induces mitochondrial permeability transition by causing the release of apoptogenic factors.²¹⁹ It is a paradox that NO can act as physiological regulator as well as a cytotoxic agent. NO mediates these activities and its main pathological derivative is formed by reacting with superoxide, to produce the peroxynitrite ion (ONOO⁻), which is a potent oxidant and cytotoxic agent. Much later it was discovered that small quantities of NO inhibited mitochondrial respiration by competing with molecular oxygen thus acting as a regulator of mitochondrial respiration.¹⁴⁵

There is increasing evidence regarding the cytoprotective properties of NO^{219 126,172,222} Rakhit and colleagues²²³ suggest that free radicals produced by NO, depending on the circumstance may mediate preconditioning. Later they presented evidence that the protection they observed using SNAP in cardiomyocytes subjected to oxidative stress was via a cGMP mechanism.¹⁷³ HSP70 has been implicated in NO induced protection

of the heart; Malyshev and colleagues²²⁰ have proposed that HSP70 is activated by NO via heat shock factor (HSF) synthesis. They hypothesise that HSF trimerisation is catalysed by the nitrozonium ion (NO^+). Another group have also reported on the upregulation of HSP70 via HSF but in smooth muscle cells.²²⁴ Kim and colleagues²²⁵ have determined that NO induction of HSP70 expression by the NO donor SNAP may possibly explain the protection afforded to hepatocytes against $\text{TNF}\alpha$ induced apoptosis. We have previously observed that the NO donor SNAP and sodium nitroprusside (SNP) are able to upregulate HSP70 as well as heme oxygenase-1 (HO-1) and cyclophilin A (CyP A) expression (abstract from Cold Spring Harbour meeting, 1998) in H9c2 cells. Consequently in this study H9c2 cells were also used. However, HSP70 induction was not observed in this study which could be due to a shorter incubation time course for H9c2. Previously, H9c2 were incubated for 6 hours in 1mM SNAP whereas in this study H9c2 cells were incubated for 2 hours.

Cyclophilins participate in *de novo* protein folding^{108,226} which leads us to believe that they may be cardioprotective. Doyle and colleagues²²⁷ demonstrated that cyclophilin A protects cardiomyocytes against oxidant induced necrotic cell death.

In these sets of experiments cardiomyocytes were exposed to SNAP for 2 hours. These results show initial protection immediately after exposure to oxidant stress that was originally thought to be partly due to HSP70 upregulation. However, Western blot analysis did not reveal any upregulation of HSP70 that could account for the observed early protection. Delayed protection was afforded to the cells 24 hours later which in part may be explained by the action of cyclophilin in conjunction with HSP70 and other heat shock proteins as was demonstrated previously (abstract from Cold Spring Harbour meeting, 1998). Once again Western blot analysis disproved this hypothesis. Western analysis instead revealed the significant upregulation of HSP75 and a slight increase in expression of HSP27.

Heat shock protein 75 is a mitochondrial protein that is a member of the HSP70 molecular chaperone protein family. It is primarily involved in unfolding proteins and

translocation of polypeptides into the mitochondrial matrix (a process which is ATP dependent) whereupon the polypeptide is released to the chaperonins (HSP60, HSP10) that assist them in refolding these peptides to their native state.²²⁸ Presumably increased expression of HSP75 may occur due to the greater requirement in translocation and folding of proteins.

The main aim of this work was to determine the effect on $\Delta\psi_m$ and cell viability of the nitric oxide donor SNAP pre-treatment on cardiomyocytes. We have been able to demonstrate that NO is able to partially restore $\Delta\psi_m$ and therefore decrease the potential to form MPTP as well as improve cell viability of cells that have been challenged by oxidative stress with the possible involvement of HSP75 activation.

Although the mechanism of this protection is not known in detail. Balakirev and colleagues report that NO reduces formation of the MPTP by reversible inhibition of cytochrome oxidase which alters membrane potential and Ca^{2+} efflux³⁰. Lowenstein speculated that NO inhibition of mitochondrial respiration might be beneficial.²²⁹ Moncada and Erusalimsky state that NO binding to cytochrome oxidase may ensure that O_2 is more available to surrounding tissue. Reduction in O_2 will cause an increase in NO production resulting in vasodilatation and increased perfusion of tissue.¹²⁸

We were interested in analysing HSP27 expression because it has been shown to play a protective role in cytoskeletal formation. It is a member of the so called small heat shock protein family (sHSP) which include $\alpha\beta$ -crystallin also upregulated in response to oxidative stress and providing protection by stabilising cytoskeletal structures.²³⁰ We previously reported that HO-1 was upregulated by NO donor pre-treatment (abstract from Cold Spring Harbour, 1998). Therefore it is very likely that upregulated HO-1 participates in the protection afforded to these cardiomyocytes.

Assessment of cell viability in this model by the trypan blue exclusion assay, which measures total cell death, (Figure 6.4) did not reveal any statistical significant protection in any of the two models. Other cell viability assays that measure plasma membrane lysis, as does the trypan blue assay, such as lactose dehydrogenase assay (LDH) release

and the fluorescent dye propidium iodide (PI) may help to clarify these discordant results. Differences in the response of H9c2 to 50 μ M H₂O₂ only, as observed in the viability assays may be a reflection of the poor condition of the cells initially although, cells were not used past passage 29 and confluency was never more than 60 – 80%. However, the data obtained from the Trypan blue assay can be quite subjective as it is reliant on the researcher visually counting all the compromised cells within a given area. Taken together, PI, LDH and Trypan blue are good assays to determine plasma membrane lysis and as such, complement the MTT assay. The MTT assay, which by determining the energetic status of the cell, may be used as a crude assay to measure apoptosis²³¹ in conjunction with the DiOC6 (3) assay.

In summary, we have reported on the cytoprotective properties of the NO donor SNAP against oxidative stress. According to FACS and MTT data, protection was at its greatest 24 hours after the initial SNAP incubation. This may result from the fact that SNAP not only liberates NO but also metal ions,²³² which after 24 hours may activate protective signalling cascades or HSP's. Elevated levels of HSP75 expression in conjunction with HSP27 may be responsible in part for the observed protection. Further studies are needed in order to ascertain the degree to which these HSP's and other factors are involved in NO induced protection

CHAPTER 7

7. OVEREXPRESSION OF CyP-D IN *IN VITRO* CELL SYSTEMS

7.1 Introduction

Mitochondria play a primary part in the onset and development of ischaemia/reperfusion injury and are recognised as major targets of toxicity, due to their key role in oxygen utilisation and ATP synthesis. It is widely accepted that mitochondrial permeability transition pore (MPTP) formation is a crucial event in ischaemia - reperfusion injury and cell death.¹³⁹ Heat shock proteins are up regulated in conditions of stress and form part of the mitochondrial chaperone complex; they are essential for protein, folding, refolding, transport and translocation through the intracellular membranes.⁶⁷

HSP70 is the most abundant and widely studied stress protein. It is one of the essential regulators of the stress response mechanism in eukaryotes. HSP75 is localised in the mitochondrial matrix where it is essential for import, folding and assembly of mitochondrial proteins.⁶⁸ The function of HSP75 is affiliated with the chaperonins, HSP60, HSP10 and cytoplasmic HSP70.⁶⁸ HSP60 is a crucial part of the mitochondrial chaperoning complex and is obligatory for normal mitochondrial biogenesis. It has been demonstrated that simultaneous overexpression of HSP60 and HSP10 confers protection against ischaemic damage, whereas overexpression of HSP60 or HSP10 separately does not offer any significant protection in rat cardiomyocytes.⁶⁹ In *Neurospora crassa*, it was recently discovered that cyclophilin 20 plays an essential role in mitochondrial protein folding in conjunction with HSP70 and HSP60.⁶⁸ We are able to demonstrate that cyclophilin expression is elevated in eukaryotic myocytes in response to heat and oxidative stress.¹⁰⁷

All cyclophilins can be thought of as editing mechanisms in the process of protein folding and we consider some of them such as cyclophilin 20 in yeast to be an essential element of the mitochondrial chaperone complex. CyP-D exists as an integral member of the MPTP and cyclosporin A can potentially inhibit MPTP formation by binding to CyP-D, thereby preventing it from binding to components of the inner mitochondrial membrane.³⁸

We are interested in CyP-D because, along with the voltage dependent anion channel (VDAC), hexokinase and the adenine nucleotide translocase (ANT), has been implicated in the formation of the MPTP^{29,44,55} leading us to the following hypothesis and null - hypothesis:

- Cyclophilins are an essential component of the mitochondrial chaperone machinery, and therefore, simultaneous overexpression of the basic complex of molecular chaperoning proteins in myocytes may afford the cells a greater degree of protection.

Null:

- The basic MPTP structure which forms, causing reperfusion injury comprises the voltage - dependent anion channel (outer membrane), cytosolic proteins (hexokinase), adenine nucleotide translocase (inner membrane) and CyP-D; hence CyP-D overexpression would cause a decrease in cell viability due to its involvement in the formation of the MPTP structure.

We suggest that the mitochondrial chaperone complex is crucial for maintaining the mitochondrial function and integrity in the process of intracellular adaptation, specifically relating to ischaemic stress. We propose that overexpressing cyclophilins will enable cells to tolerate ischaemia/reperfusion injury, because the increase in this molecular chaperone means that a greater amount of denatured protein is being refolded thereby preserving the structure and function of the mitochondrion.

7.2 Methods

7.2.1 Cell Culture

COS-7 and human embryonic kidney 293 (HEK 293) cells were obtained from the American Type Culture Collection (CRL-1446, ATCC Rockville, MD, USA) These were grown until half confluent for transfection in full growth medium (FGM), Dulbecco's Modified Eagle Medium (DMEM) with sodium pyruvate and 1000mg/l glucose (Gibco-BRL) and 10% (v/v) foetal calf serum in 95% atmosphere and 5% CO₂.

7.2.2 Primary rat neonatal cardiomyocytes

Chapter 2 offers a full description of primary rat neonatal cardiomyocyte isolation

7.2.3 Plasmid Construction and Transfection

The CyP-D gene was a gift from Dr D.Bergsma and was subcloned into pBluescript. After its orientation was ascertained via restriction mapping, it was further subcloned into the expression vector pcDNA3 (designated pcDNA3-CyP-D) in the correct orientation for expression into the BamHI and HindIII site. CyP-D was transfected into 3 day old isolated primary neonatal cardiomyocytes using a novel integrin transfection method^{138,233} in the order as shown in Figure 7-1. Green Fluorescent Protein (GFP) in the pCAGGS vector (designated pCAGGS-GFP) was used as a control for transfection efficiency. A second control consisted of the vector pcDNA3 with no DNA. (A more detailed description is available in Chapter 2).

7.2.4 SDS – PAGE and Western blotting

Cells were harvested into 200µl (2x) concentrated SDS-PAGE sample. The samples were loaded into 4% stacking gel and separated by electrophoresis on a 12% running gel. The proteins were transferred at 30V overnight into nitro-cellulose membrane (Hybond-C, Amersham, UK) and probed with anti-cyclophilin A (used as a general cyclophilin antibody, it was thought that any increase in CyP expression could be accountable to CyP-D as this was overexpressed and not CyP-A due to the non availability of a specific CyP-D antibody) at a concentration of 1:1000 in PBS (phosphate buffered saline) with 0.05% Tween 20 in 0.1% Marvel. Using peroxides-conjugated rabbit anti-mouse antibody (DAKO), the blot was conjugated at a dilution of 1:2500 and detected using enhanced chemiluminescence (ECL: Amersham, UK).

7.2.5 Lethal Simulated Ischaemia / Reperfusion (LSI/R)

Cells in 12 well tissue culture plates (Nunc) washed once with PBS followed by the addition of 1ml SI buffer (as described in Chapter 2). The culture vessel containing the cells were then transferred to anaerobic GasPak pouches (Becton Dickinson, Sparks Md) and incubated at 37°C for 1 hour and reperfused for 1 hour. The pouches were pre-gassed with 5% CO₂, 95% Argon and the O₂ content in these bags was <1% during the experiment and was monitored by an anaerobic indicator.

7.2.6 Isolation of Mitochondria from Cells

Transfected and non-transfected 293, COS-7 and primary neonatal cardiomyocytes cells were grown in 35x 10cm petri dishes were harvested and resuspended in MSMEB (210mM mannitol, 70mM sucrose, 10mM MOPS, 1mM EGTA and 0.5% BSA). The

following procedures were all performed at 4⁰C. Digitonin (5% in DMSO) was added until >90% cell permeabilisation occurred as confirmed by Trypan blue exclusion. After the addition of a further 1ml MSMEB the samples were spun and the supernatant removed. The pellet was homogenised 10 times in a dounce homogeniser. The supernatant was spun at 2500g for 3 minutes and finally 10 minutes at 12000g. The resulting pellet (mitochondrial fraction) was washed along with the supernatant (cytosolic fraction). ²³⁴ Protein concentration was calculated using a Bio-Rad protein assay.

7.2.7 Determination of Peptidyl - Prolyl isomerase (PPI-ase) Activity

After the mitochondria were freeze- thawed as previously described (section 2.2.1). They were resuspended in 35mM HEPES buffer (generally 100 μ l). From the lysate the protein concentration was ascertained (Biorad protein assay). Generally 20 μ g protein was used in each assay. HEPES buffer, α -chymotrypsin, mitochondrial lysate was added to a cuvette to volume of 990 μ l. They were cooled to 4°C, 5 minutes before the addition of 10 μ l of the peptide N-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide at a concentration of 8mM. This initiated the reaction that was recorded over two minutes on a spectrophotometer (Helios α , Unicam Ltd., Cambridge, U.K.) at an absorbance of 390nm at 4°C. Each sample was assayed three times in order to determine an average of the PPIase enzymatic activity that was finally calculated by an enzymatic data fitting computer software package (GraFit). This method was taken from Kofron and colleagues.¹³⁴

7.3 Results and Discussion

Primary rat neonatal cardiomyocytes were seeded into 6 well plates and transfected in the order as indicated in Figure 7.1 with either pCAGGS-GFP (positive control) or pcDNA3-CyP-D plasmids. After transfection cells were subjected to 6 hours SI with 2 hours reperfusion.

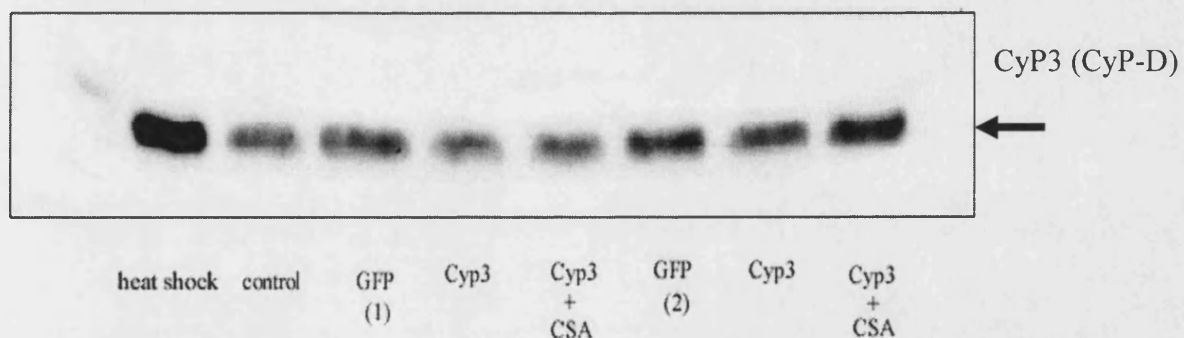
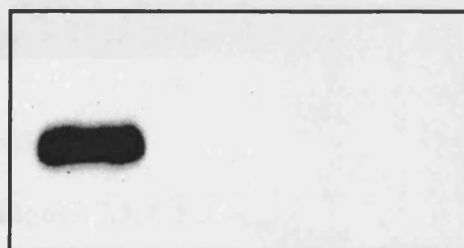


Figure 7-1 Western blot of transfected cells.

This Western blot shows expression levels of CyP-D in neonatal cardiomyocytes transfected with the vector pcDNA3-CyP-D; pCAGGS-GFP; control untransfected cells; heat shocked sample acting as positive control and pcDNA3-CyP-D with 0.2 μ M

CsA. Transfected CyP-D samples were not significantly different from the control. CsA treated samples also do not significantly affect CyP-D expression levels. Only the positive heat shock samples are significantly different from the control. GFP (1) and GFP (2) refer to the different batches of GFP used as mentioned below.

Two days following transfection, GFP expression was detected using a fluorescence microscope (Figure 7.3). Two differently prepared batches of GFP were used (Batch 1 and Batch 2). According to fluorescence analysis, Batch 1 fluoresced more intently. The difference between the two became more evident in the Western blot (Figure 7.2). Binding of GFP antibody was only evident in Batch 1.



GFP (1)

GFP (2)

Figure 7-2 Western blot demonstrating two different batches of GFP.

GFP (1) was used in the transfections as these blots demonstrated its correct functioning

*CyP3 =CyP-D

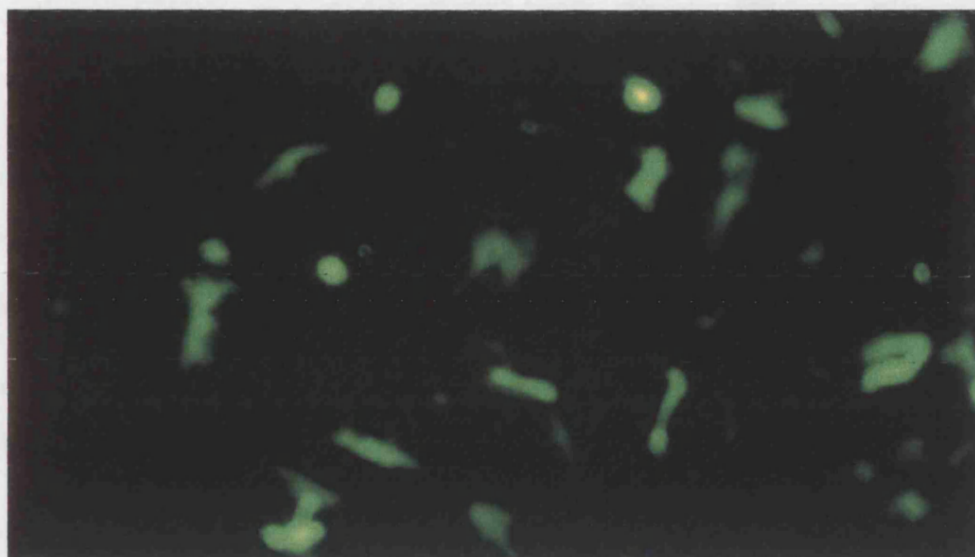


Figure 7.3. GFP expression as visualised by fluorescence microscopy

The Cyclophilin probed blots Figure 7.1 did not demonstrate any overexpression of CyP-D when compared with the control and heat shock samples, which suggested that these cells were not properly overexpressing CyP-D.

We hypothesised that cells in wells which had been transfected with CyP-D and subjected to oxidative stress would have a lower survival rate, since CyP-D is believed to form the MPTP that leads to cell death, in comparison to cells not transfected with CyP-D. Transfection of CyP-D had no effect on COS-7 cells as determined by functional assays (MTT) Figure 7-11A. However, the PPIase assay demonstrated statistical significant difference when compared to the control. We also re-transfected neonatal cardiomyocytes and increased the amount of DNA used (4-8 μ g), but this also had no effect. It is possible that the transfection may have been successful but there was a possibility that the non-specificity of the cyclophilin antibody to CyP-D was unable to detect upregulation.

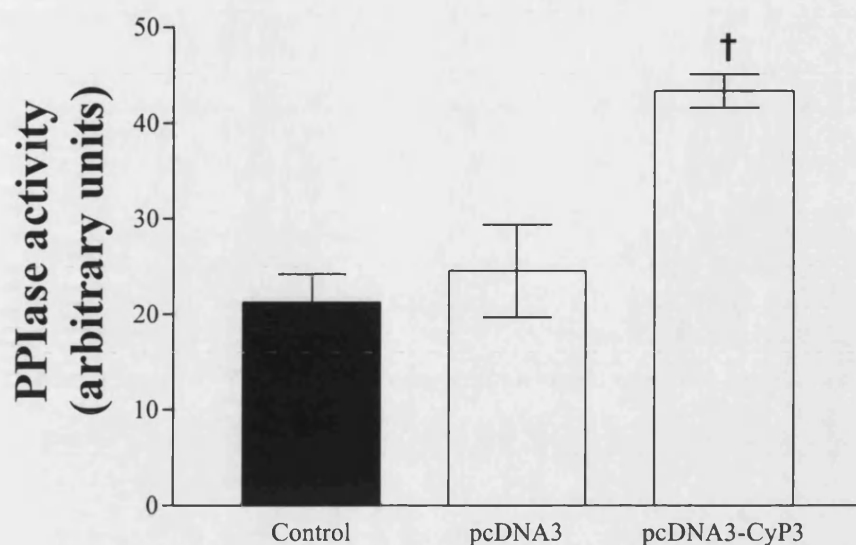


Figure 7-4 PPIase activity of neonatal cardiomyocytes transfected with CyP3

Primary rat neonatal cardiomyocytes were transiently transfected with pcDNA3-CyP3 or pcDNA3 without an insert (sham). Mitochondria were isolated from control, pcDNA3 and pcDNA3-CyP3 transfected cells and activity was determined in each of the three groups. CyP3 transfected cells show a significantly increased level of activity as opposed to the control. The PPIase activity was measured at least three times in each group (mean \pm SEM) Statistical significance of difference was compared with control. †P<0.05

PPIase assays were conducted on the transfected samples as another mode in which to determine if the transfection was successful. The CyP-D insert possesses a sequence that targets it to the mitochondria,¹⁰⁹ and as this assay works on isolated mitochondria, where only CyP-D is present. Therefore any increases in PPIase activity when compared to the control will be due to the transfected CyP-D. Figure 7-4 demonstrates the detectable increase in the PPIase activity of the transfected samples in primary neonatal cardiomyocyte cells. It was quite apparent that in primary cardiomyocytes overexpression was only detectable in PPIase activity assays (Figure 7-4). Western analysis of control and vector transfection only in HEK 293 cells (Figure 7-6) still revealed no detectable overexpression, possibly due to the non-specificity of the cyclophilin antibody. The results from the previous experiments have led us to believe that the problems we were experiencing may be because of:

1. Contamination of the DNA insert, CyP-D, by protein, phenol.
2. The CMV promoter of the pcDNA3 vector did not work effectively in primary rat neonatal cardiomyocytes cells.
3. Non-specificity of the cyclophilin antibody for the mitochondrial CyP-D

In order to circumvent these problems, we cleaned the CyP-D and pcDNA3 vector using a caesium chloride gradient (as described in Chapter 2) and transfected into human 293 commercially available cell lines which are known to have high transfection efficiencies, and express pcDNA3. There are many publications demonstrating the effectiveness of the CMV promoter in pcDNA3 when transfected into COS-7 and 293 cell lines.^{235,236}

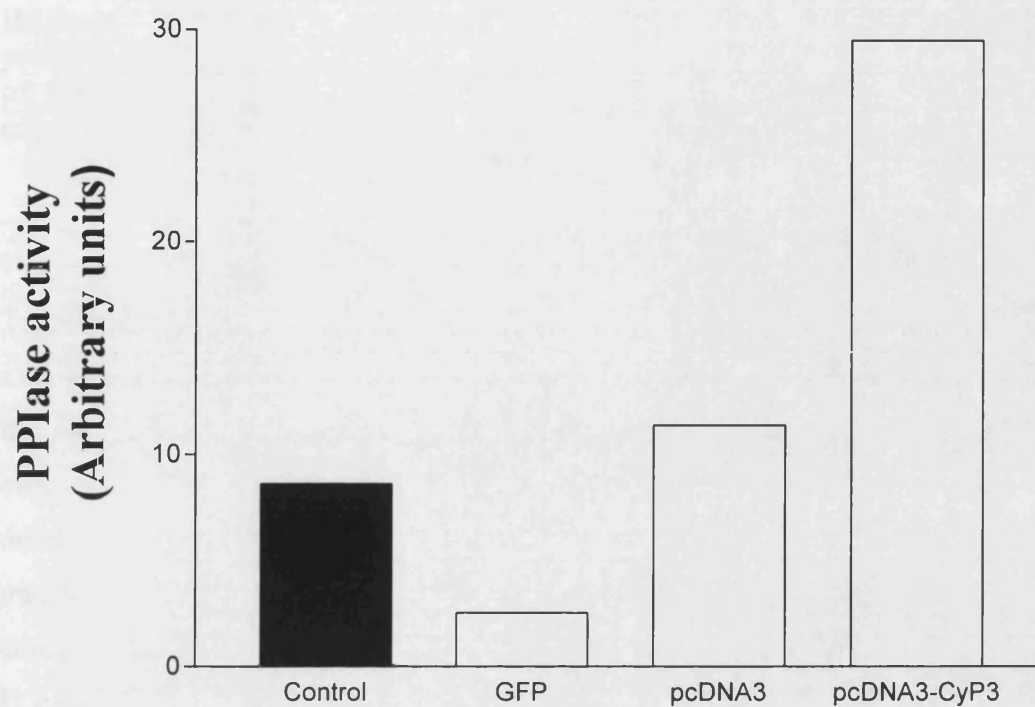


Figure 7-5. Effect of overexpression of CyP-D in HEK 293 cells.

Mitochondria from HEK 293 cells were extracted and analysed for differences in PPIase activity. This is another method of comparing whether or not CyP-D was successfully overexpressed in cells when compared to control and samples containing vector alone (pcDNA3).

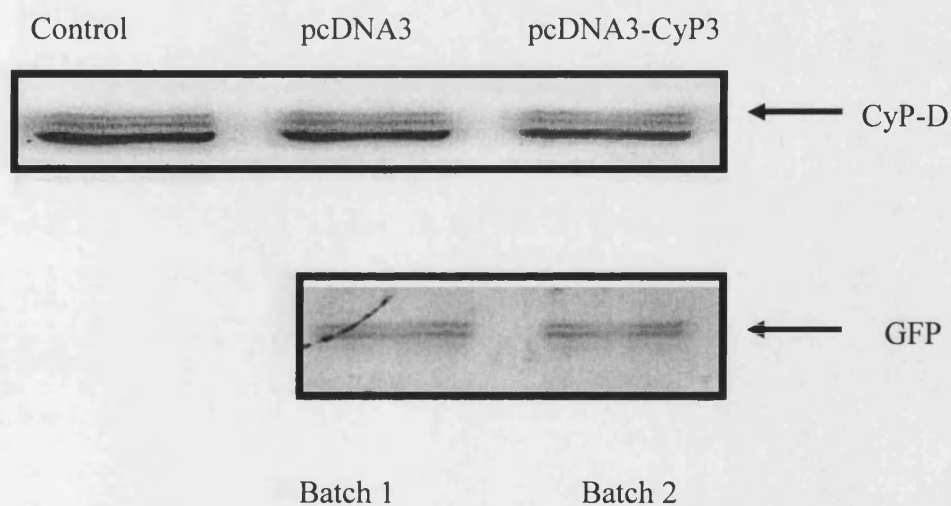


Figure 7-6 Western blot of HEK 293 cells transfected with pcDNA3, pcDNA3-CyP3 and pCAGGS-GFP

HEK 293 cells were transfected with pcDNA3, pcDNA3-CyP3 and pCAGGS-GFP and protein was isolated and probed with a cyclophilin and GFP antibody. Overexpression of CyP3 was not detected from protein isolates although GFP transfection control expression is visible.

Transfection into these cell lines would test the possibility that the inability to detect the overexpression by Western blot protocol, would indicate that the problem may lie with the cyclophilin antibody. The 293 cells were harvested post transfection for Western blotting (Figure 7-6) and PPIase activity assay (Figure 7-5). Overexpression was only detected by the PPIase method only (Figure 7-5). Another problem with this transfection protocol was that the positive control and CyP-D insert were on two completely different vectors, so the GFP was not an accurate or direct control for CyP-D expression. Furthermore, we know from previous work that cDNAs (such as GFP) are efficiently expressed from the pCAGGS vector in cardiomyocytes.²³⁷

A redesigned vector was provided by Martin Goss (a colleague at the Rayne Institute) (Figure 7-7) that has the pCAGGS vector backbone with the GFP control gene and multiple cloning sites already incorporated. It therefore has an β -actin promoter and in addition a CMV enhancer to drive the expression of the CyP-D and GFP. The original pCAGGS vector with GFP subcloned into it worked well as a control in all the cells it was transfected into. Therefore we knew there should not be any problems with the β -actin promoter on the new vector. If the downstream GFP is expressed then CyP-D

should be overexpressed as the same promoter drives them both on this vector.

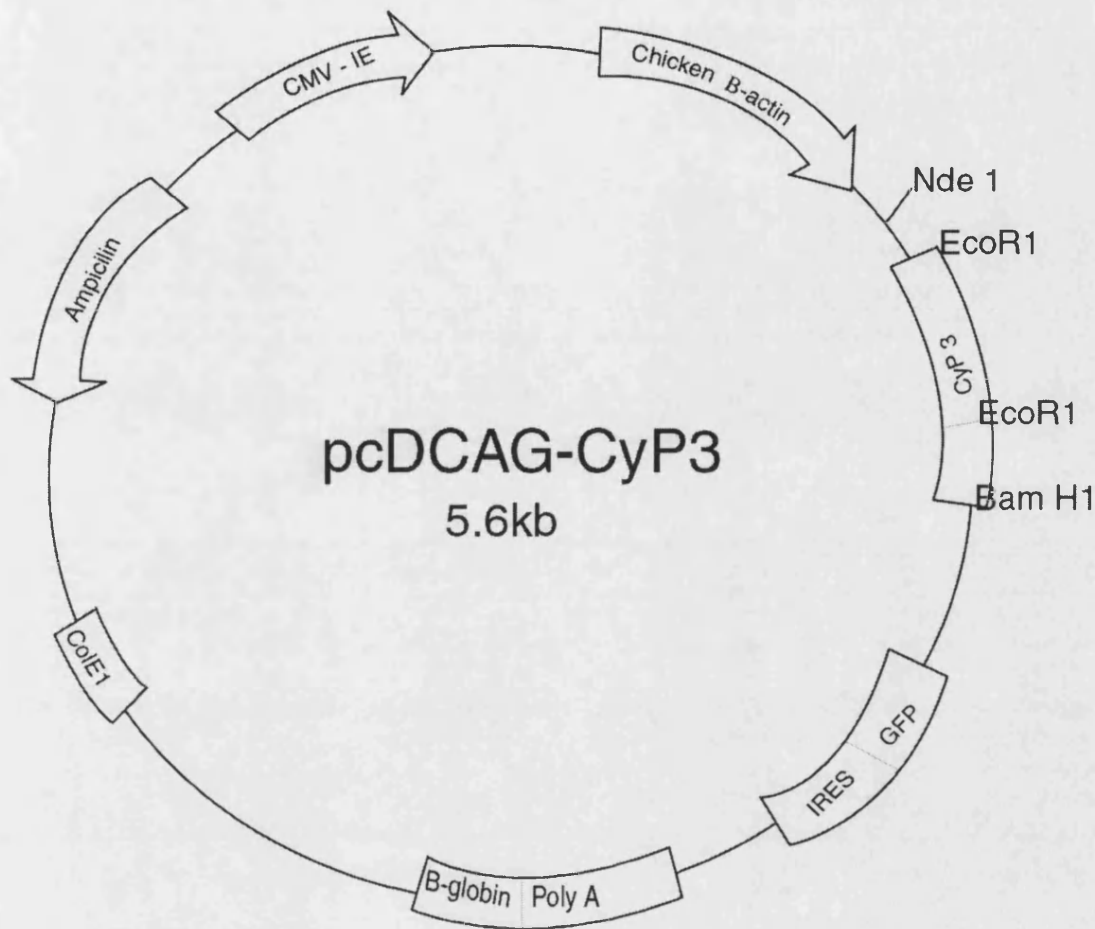


Figure 7-7. Schematic representation of the redesigned plasmid (pcDCAG-CyP3) provided by Martin Goss.

GFP and the coding region of CyP-3 are under the control of chicken β -actin promoter and immediate early enhancer of cytomegalovirus. GFP was co-expressed with the gene of interest using an IRES (internal ribosome entry segment) expression cassette.

This vector was initially transfected into COS-7 cells, as this was the cell line the integrin transfection protocol was originally used in. After 48 hours GFP expression was visualised on the fluorescence microscope, which were later harvested for Western blotting (Figure 7-9A) and PPlase activity (Figure 7-8) was accessed. After probing with the cyclophilin antibody the blot was re-probed with GFP antibody (Figure 7.9B).

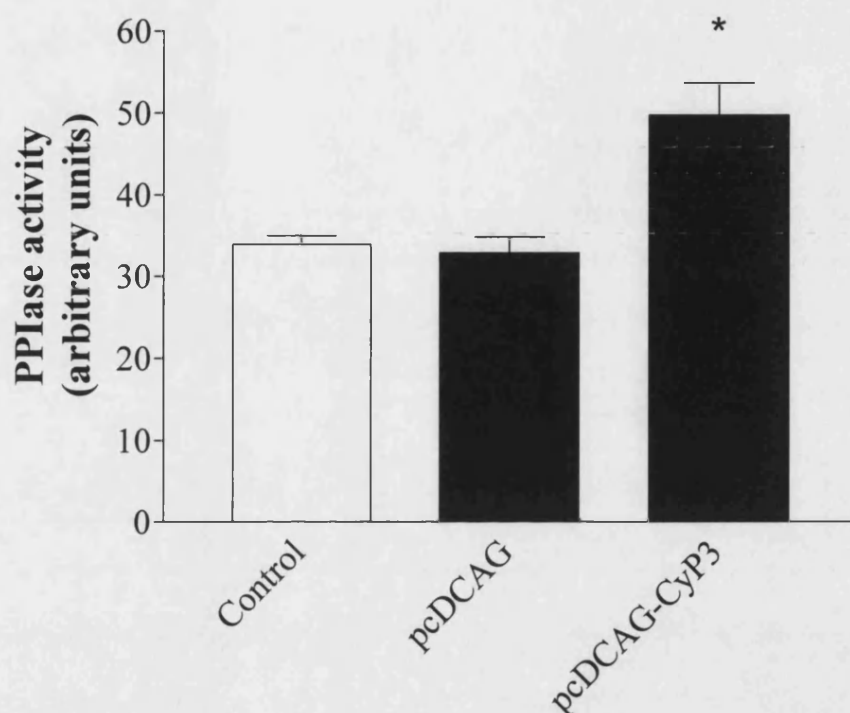


Figure 7-8. Transfection of pcDCAG-CyP3 in COS-7 cells

Transient transfection into COS-7 cells results in an increase in PPIase activity. Cells were transiently transfected with pcDCAG-CyP3 in order to test the efficacy of this new construct. These cells were cultured until confluent (usually 2 days after transfection) and harvested for protein and mitochondrial isolation. PPIase activity was estimated as described previously. Bars represent S.E.M of triplicate samples. The PPIase activity of pcDCAG-CyP3 is significantly different from that of the control and (vector without CyP-3 insert) pcDAG sample at * $P < 0.05$ v control.

In Figure 7-9A Western blot analysis of the transfectants using the protein lysate isolated from the above transfection with the new expression vector, demonstrates that there is no increase in CyP-3 when probed with cyclophilin antibody. Although when probed with GFP antibody, expression of GFP is present (Figure 7-9B) which means that if GFP was transcribed then CyP-3 should also have been expressed.

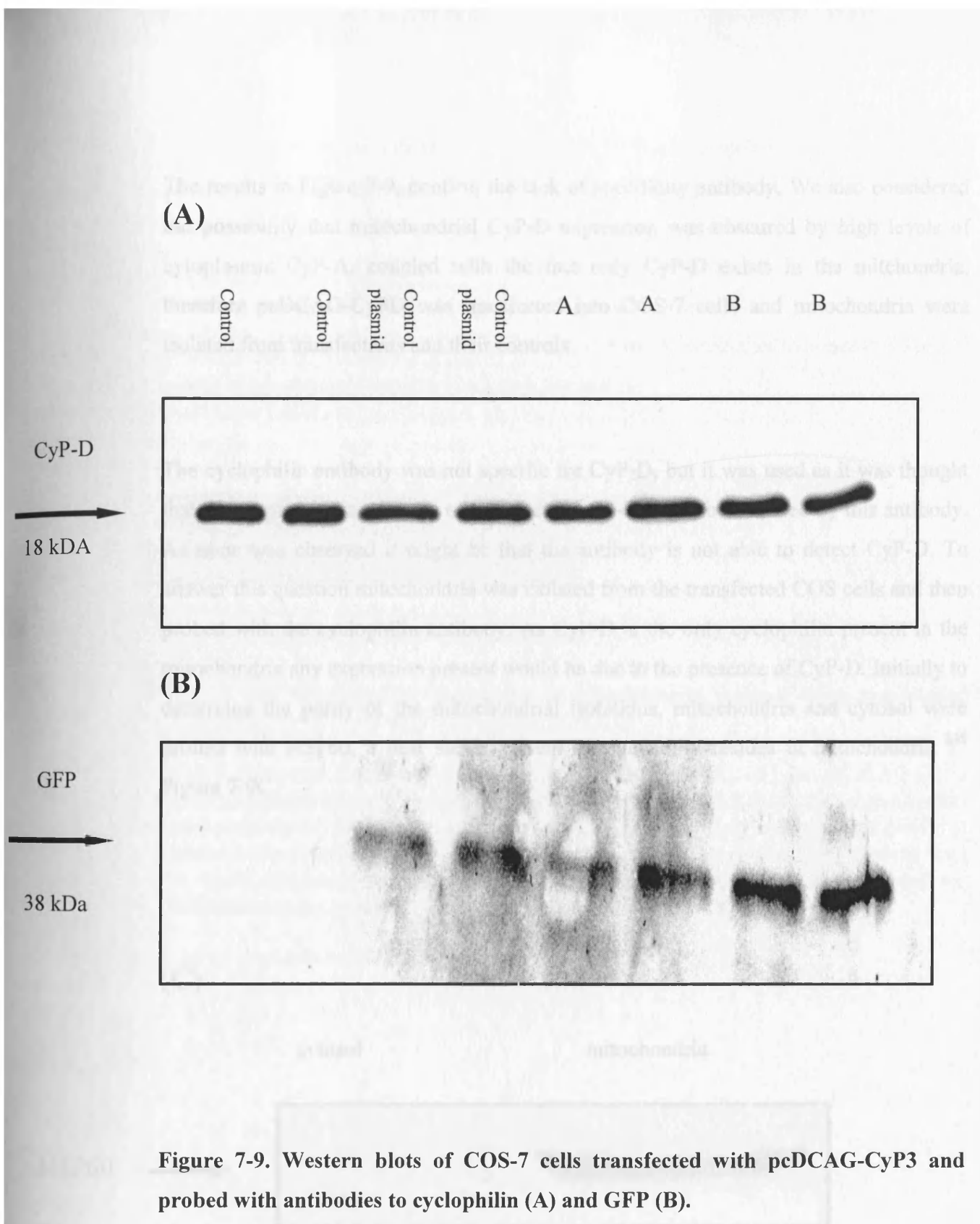


Figure 7-9. Western blots of COS-7 cells transfected with pcDCAG-CyP3 and probed with antibodies to cyclophilin (A) and GFP (B).

Western blot results (**Figure 7-9**) confirmed GFP (**B**) expression but no overexpression of CyP-D was visible (**Figure 7-9A**). (Two different aliquots of CyP-D were prepared and labelled as A and B, and transfected in duplicate as indicated in **Figure 7-9A**).

Western blot demonstrating HSP60 expression, which was used to confirm the purity of mitochondrial isolations from COS-7 cells, as HSP60 generally, exists in mitochondria.

The results in Figure 7-9, confirm the lack of specificity antibody. We also considered the possibility that mitochondrial CyP-D expression was obscured by high levels of cytoplasmic CyP-A, coupled with the fact only CyP-D exists in the mitochondria, therefore pcDCAG-CyPD was transfected into COS-7 cells and mitochondria were isolated from transfectants and their controls.

The cyclophilin antibody was not specific for CyP-D, but it was used as it was thought that overexpression as a result of transfected CyP-D might be detected by this antibody. As none was observed it might be that the antibody is not able to detect CyP-D. To answer this question mitochondria was isolated from the transfected COS cells and then probed with the cyclophilin antibody. As CyP-D is the only cyclophilin present in the mitochondria any expression present would be due to the presence of CyP-D. Initially to determine the purity of the mitochondrial isolations, mitochondria and cytosol were probed with HSP60, a heat shock protein that generally resides in mitochondria²³⁸ Figure 7-9C.

(C)

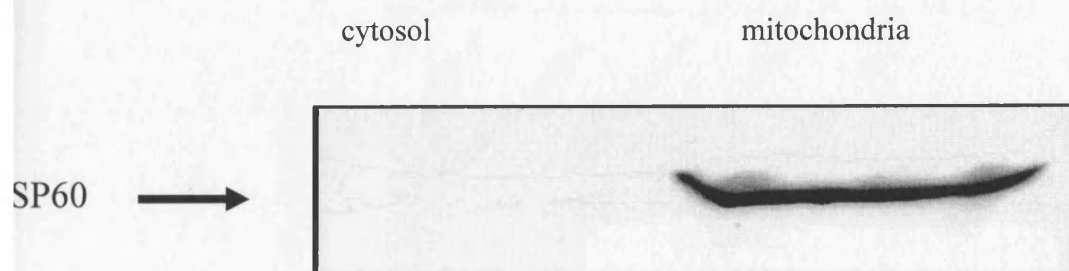


Figure 7-9C. HSP60 expression in the mitochondrial fraction from CyP-D transfected cells,

Western blot demonstrating HSP60 expression, which was used to confirm the purity of mitochondrial isolations from COS-7 cells, as HSP60 generally, exists in mitochondria.

The mitochondrial isolates were then analysed by Western blotting. They were probed with the cyclophilin antibody (Figure 7-10) as performed previously. CyP-D expression was detectable at 20 kDa implying that this antibody was able to detect the protein expression of this cyclophilin subset. The protein expression of the transfected samples however, was no different from that of the non-transfected controls. Nevertheless, as the PPIase assay revealed significant differences between transfected and non-transfected samples, we decided to conduct cell viability assays.



*CyP+ Control pcDCAG pcDCAG-CyP3

Figure 7-10 Western blot expression of mitochondria isolated from transfected COS-7 cells.

COS-7 cells were transfected with the control plasmid (pcDCAG) and pcDCAG-CyP3 vector. Mitochondria were harvested from these transfected cells and the protein lysates were probed with cyclophilin antibody. As CyP-D is the only cyclophilin subset that resides in the mitochondrion therefore any protein expression derived from probing with the CyPA antibody should be CyP-D. The Western blot in this Figure does not demonstrate that CyP-D has been overexpressed in these cells

*CyP+ = cyclophilin positive control

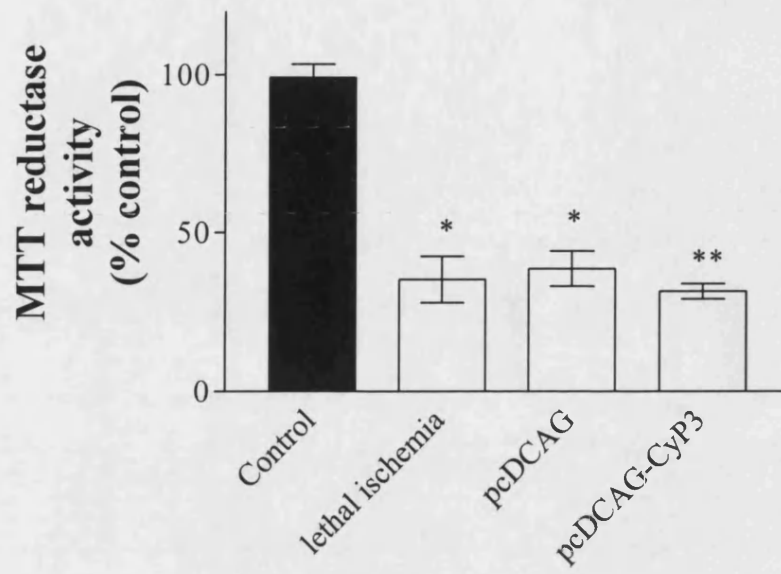
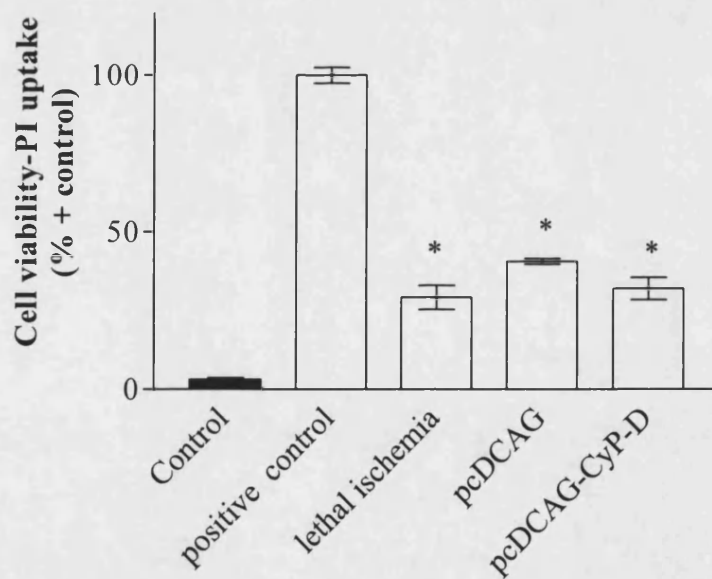
A**B**

Figure 7- 11. Cell viability assays COS-7 cells transfected with pcDCAG-CyP-D.

These graphs display that the CyP3 transfectants had no effect on survival of COS-7 cells that were subjected to 1 hour SI/R as determined by MTT (A) and PI (B) assays

when compared to non-transfected control and positive control **(B)**. Positive control were COS-7 cells, which were freeze-thawed in liquid N₂ to rupture cell membranes prior to incubation with PI solution.

7.4 Conclusion

We have attempted to solve the problems that we have encountered with the transfection procedure by re-extracting and purifying the plasmid with the CyP-D insert. The cyclophilin antibody was not able to recognize CyP-D overexpression. We concluded that the transfections were working with the new vector due to the consistent positive results from the PPIase assay. However, RT-PCR did not reveal overexpression of CyP-D. These are classical methods of determining overexpression of a gene and until we are able to achieve this we cannot evaluate any results we obtain from these assays.

It may have been possible to use other strategies such as creating a stable cell line overexpressing CyP-D, which would have meant that, repeated time consuming transfections would not have been necessary, and there would always be a constant renewable source of transformed cells. The CyP-D insert could have been subcloned into other vectors, which we have subsequently demonstrated to work well in cardiomyocytes. A major problem we encountered was the possible non-specificity of the cyclophilin antibody used, which is why we conclude we could not detect overexpression. Our attempts to produce a specific polyclonal antibody were unsuccessful. However, based on the PPIase activity assay data, we can say that the transfection was successful, and therefore more assays should be conducted to ascertain the effect of overexpressing CyP-D in cardiomyocytes. However, the initial data we have obtained from the viability assays that have been conducted with COS-7 cells. Suggest that overexpression did not afford the cells any protection (Figure 7-10). Based on these results it would be interesting to repeat these mitochondrial isolations in CyP-D transfected neonatal cardiomyocytes.

The concept of gene transfer has had a great impact on biological research. Numerous studies have demonstrated the protective effect that transfection of heat shock proteins confers to cardiac cells.^{69,133,145,239-241} The use of adenovirus-mediated transfections into human myocardium may be impractical because of the possibility of mutation to active

forms and their immunogenic properties. Cationic lipids are the preferred reagents for transfer of genes because of their simplicity, high transfection efficiency and low safety risks. With the advancement of gene therapy technology, it may only be a matter of time before HSP's are used to afford protection to patients undergoing heart bypass surgery and other procedures.

CHAPTER 8

8. GENERAL DISCUSSION

8.1 Overview

Ischaemia / reperfusion injury induces cell death, but the mechanisms that are critical for causing cell death are not known. In this thesis I sought to clarify the relationship between cyclophilins (specifically mitochondrial CyP-D) and mitochondrial heat shock proteins (mHSP) HSP75, 60 and 10 in this phenomenon. The aims of this thesis are to explore the role of cyclophilin and the mHSP's in myocardial ischaemia /reperfusion injury. This thesis is based on the overall hypothesis:

“Cyclophilins are components in the formation of the MPTP and therefore we state that they play a major role in the cell death pathway”.

This hypothesis has been investigated using 5 approaches. These involved using an *in vitro* lethal simulated ischaemia/reperfusion (LSI/R) model to investigate effects of ischaemia/ reperfusion (IR) injury on the development of apoptosis and necrotic cell death. A buffer and nitric oxide donor S nitroso N acetylpenicillamine (SNAP) were used to precondition cells in another two chapters in order to investigate cyclophilins and the mHSP's as well as mitochondrial membrane potential ($\Delta\psi_m$). The expression of the HSP's was investigated in subsarcolemmal (SSM) and interfibrillar (IFM) mitochondria in female rats, which were subjected to left anterior descending (LAD) coronary artery occlusion and reperfusion for predetermined periods. Finally CyP-D was transfected into cells in order to determine whether it was deleterious or afforded cell protection during IR.

Myocardial IR is a complex phenomenon that induces cell damage through a biphasic process. Ischaemia initiates the injury by deprivation of the energy process needed to

maintain ionic gradients and homeostasis. Reperfusion exacerbates this damage by generation of deleterious agents such as free radicals. Various techniques and models have been used in this thesis to investigate the different theories proposed.

8.2 Discussion of Methodology

8.2.1 LSI/R and SI/R model

In Chapter 3 a cellular model of lethal simulated ischaemia/reperfusion (LSI/R) was established. This *in vitro* model attempted to mimic conditions that occur during myocardial IR. Rat neonatal cardiomyocyte cells were incubated in the ischaemic buffer, which attempted to mimic conditions such as high salt concentration, acidosis, substrate deprivation, inhibition of mitochondrial respiration and low oxygen tension. Similar ischaemic buffers are used to generate what is termed “chemical hypoxia” aptly named because chemical compounds such as sodium dithionite¹⁸⁷ or amobarbital²⁴² are used that inhibit mitochondrial respiration and so aid in simulation of myocardial ischaemic conditions. Some researchers also employ the use of systems to lower pO₂ in addition to ischaemic buffers to lower oxygen tension to between 1 to 8mmHg.^{22,243} In our model as described in Chapter 2, a hypoxic chamber was used to lower pO₂ to 2mmHg, which was maintained throughout the course of the experiment. Experiments with a Clark type oxygen confirmed that the buffer alone did not maintain hypoxic conditions for the duration of the experiments. Using the chamber ensured that hypoxia as we defined it (~2mmHg) was maintained. The conditions produced were termed lethal simulated ischaemia/reperfusion (LSI/R). A milder form was named simulated ischaemia/reperfusion (SI/R) and was used in Chapter 4 in which the chamber was not used. This was due to the fact that the cells were incubated in the SI buffer for short periods that is adequate to precondition cardiomyocytes; however, LSI/R was used as a severe insult to the cells to determine protection conferred by preconditioning.

Cellular models of myocardial IR are not generally accepted (even though they are not complex and can be easily manipulated) because some of the adopted endpoints cannot be extrapolated to *in vivo* models,⁹⁷ and the time course of ischaemia needed to inflict

myocardial damage has no clinical relevance.⁶ These endpoints are invariably achieved by hypoxia using markers, which are substitutes for tissue infarction. Hearse and Sutherland²⁴⁴ state that these models are inexpensive and generate highly reproducible data but also admit their low clinical relevance. There are many types of preconditioning models, which are cell^{77,189,237,245-247} or animal based^{98,185,248} using isolated perfused hearts.¹⁸⁸ In spite of their reservations expressed above, large amounts of data generated from these cell-based models are supported by data from *in vivo* models. Moreover, it is recognised that these *in vitro* models are excellent for studying signal transduction pathways that are involved in many aspects of cardiovascular disease.

The cells used in these models are quite diverse. Mature adult cardiac myocytes are commonly used but are resistant to transfection. Hence many researchers use immature or dedifferentiated cardiomyocytes, which explains the rationale for using rat neonatal cardiomyocytes in Chapter 7. Using human cardiomyocytes is a distinct advantage but has obvious ethical issues. Although these systems may not reflect exactly what occurs *in vivo*, they give an idea of the mechanisms involved. Ultimately the true test bed for any therapeutic advance has to be in some form of human clinical trials.²⁴⁹

8.2.2 Left Anterior Descending (LAD) Coronary Artery Occlusion model

This is a commonly used *in vivo* model to study myocardial ischaemia/reperfusion injury²⁵⁰ as well as preconditioning studies.^{97,98} Various ischaemic and reperfusion time courses were employed as described in Chapter 5 in the study of subsarcolemmal and interfibrillar mitochondria characteristics.

8.2.3 Flow Cytometry

Flow cytometry is a technique that measures fluorescence and scattered light from the analysed particles. It differentiates between size and cell granularity and is able to differentiate between viable and non-viable cell populations. Throughout this thesis flow cytometry has been used in conjunction with propidium iodide (PI) to measure cell

viability and mitochondrial membrane potential was measured by retention of the lipophilic dye DiOC₆(3).

8.2.3.1 Mitochondrial membrane potential ($\Delta\psi_m$)

The cessation of blood flow followed by a reperfusion periods is detrimental to many cell structures. Loss of energy, alteration of ionic homeostasis, accumulation of H⁺, generation of ROS and Ca⁺ are the complex cascade of events, which are induced. In this context, mitochondria as key components of the cell are susceptible to injury and are known to modulate cell death.⁴¹

Energy is produced in mitochondria in the form of ATP by oxidative phosphorylation, which is coupled to electron transport. Electron transport chain enzymes catalyse the transfer of electrons from NADH to O₂. An electrochemical gradient is generated from energy released through electron transport driving protons across the inner mitochondrial membrane. This gradient, which is known as the proton motive force (pmf), drives the formation of ATP from ADP (using F₁F₀ ATPase). The mitochondrial membrane potential is the major component of the pmf.^{25,43} The maintenance of $\Delta\psi_m$ is a necessary requirement for normal function of cardiomyocytes and collapse of the $\Delta\psi_m$ has been implicated in ischaemia/reperfusion injury due to the formation of MPTP.^{50,156} Therefore, mitochondrial membrane potential ($\Delta\psi_m$) is indicative of the functional or energisation state of the mitochondrion,^{251,252} and as such, changes in the $\Delta\psi_m$ are integral to the life – death transition in a cell.³⁰ Maintenance of the $\Delta\psi_m$ is not only a requirement for ATP synthesis; it also drives the uptake of Ca²⁺ into the mitochondria by the Ca²⁺ uniporter. This is generally regarded as the signal for increased energy demand by the cell that stimulates ATP production.²⁵³ Due to the importance of $\Delta\psi_m$, lipophilic fluorescent cations that accumulate in the mitochondrial matrix in relation to the $\Delta\psi_m$ are commonly used controversial indicators of $\Delta\psi_m$ in cardiomyocytes. DiOC₆(3) is the indicator used in this thesis. It was used according to the recommendations of Rottenberg and colleagues who specify that using this dye at high concentrations (40-100nM), the plasma membrane potential ($\Delta\psi_p$) and not $\Delta\psi_m$ is measured and these

concentrations also inhibit cell respiration to the same degree as rotenone. To avoid this, he recommends using concentrations $<1\text{nm}$,¹³⁶ which also reduces the risk of quenching of the mitochondrially bound membrane dye DiOC₆ (3) as long as the concentrations are at subnanomolar proportions. He also used the uncoupler CCCP to collapse the $\Delta\psi_m$ at low concentrations of $5\mu\text{M}$ where the plasma membrane ($\Delta\psi_p$) was not affected as determined by DiOC₆ (3). High concentrations of $50\mu\text{M}$ resulted in both the collapse of $\Delta\psi_m$ and $\Delta\psi_p$.¹³⁶ In these experiments, we used CCCP as a positive test to determine that the $\Delta\psi_m$ was accurately measured by the lipophilic dye DiOC₆ (3). However, we used concentrations of up to $500\mu\text{M}$ CCCP. Flow cytometry analysis in conjunction with propidium Iodide (PI) allows viable and non-viable cells to be easily distinguished. Using this in combination with mitochondrial dye DiOC₆ (3) would have helped to ensure that the change in DiOC₆ (3) fluorescence was based on viable cells only.

Other researchers argue that this is not the optimum dye to use; they instead state that 5,5', 6,6' –tetrachloro-1, 1', 3,3' –tetraethylbenzimidazolcarbocyanine iodide (JC-1) is a better choice,^{251,254} while some disagree and recommend the use of rhodamine 123.²⁵⁵ JC-1 unlike most other fluorescence dyes emits light at red and green wavelengths with both emissions indicating changes in $\Delta\psi_m$. Rhodamine 123 is a single-emission dye which at high concentrations may inhibit mitochondrial respiration, and undergo self-quenching in mitochondria.¹⁴⁴ Mather and colleagues have noted that there was no change in rhodamine 123 fluorescence in response to $\Delta\psi_m$ depolarisation and recommend using JC-1.²⁵¹ Due to the controversy over the use of these dyes, it would appear that the best course of action would be to use protocols that can confirm the observed data, in other words have positive controls integrated into the experimental protocol. In this study, CCCP an uncoupler of oxidative phosphorylation stimulating mitochondrial F₁F₀ –ATPase, causing ATP hydrolysis with resultant depolarisation of the $\Delta\psi_m$ ⁴⁴ was used to ensure that $\Delta\psi_m$ was measured accurately by DiOC₆ (3) in subsequent experiments as performed by other authors.^{204,255}

Laser scanning confocal microscopy in conjunction with the fluorescent probes calcein and tetramethylrhodamine methylester (TMRM) was used to directly measure formation

of MPTP by Lemasters and coworkers.⁴⁴ Calcein normally remains in the cytoplasm and TMRM labels polarized mitochondria. Upon subjection to oxidative stress, mitochondria filled with calcein and TMRM fluorescence decreased. These events were indicative of the formation of MPTP and $\Delta\psi_m$ depolarization respectively as redistribution of calcein was blocked by cyclosporin A, they concluded that pore opening was CsA sensitive.⁴⁴ Griffiths and Halestrap devised a model in isolated mitochondria in which loading radiolabeled 2-deoxy [3H] glucose in perfused hearts, they were able to demonstrate pore opening following ischaemia/reperfusion.¹⁰⁶ Another more recent technique used by this group measures pore opening by measuring mitochondrial swelling as a decrease in light scattering recorded at 520nm on a spectrophotometer. These methods allow “real time” visualization of MPTP formation as opposed to the use of indirect and controversial mitochondrial membrane potential dyes.

8.2.4 Biochemical Assays

The MTT assay is based on the reduction of the yellow (3,4- 5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide into a blue formazan colour. This reaction is dependent on mitochondrial reductase activity linked to NADPH and NADH dependent redox systems and is thus a good method for determining cellular metabolic status.¹⁴⁸ The MTT assays in Chapter 3 demonstrate a decrease in viability that can be used as indicator of the potential of a cell to undergo apoptosis. This in combination with a cell viability assay such as lactose dehydrogenase assay (LDH), trypan blue or propidium Iodide (PI) which determine plasma membrane lysis (necrosis). In Chapter 3, Figure 3-5A, LDH release from cardiomyocytes undergoing LSI is similar to the control untreated sample. The comparable MTT assay for cardiomyocytes undergoing 3 hours LSI only (Figure 3-5B) reveals that the metabolic activity of these cells has decreased by more than have in comparison to the control. It is possible that these cells may be undergoing apoptosis as opposed to necrosis as long as they possess enough residual energy to initiate this energy dependent process. In this manner, this assay can be used as a crude method to measure apoptosis, although the data would be more robust if used in conjunction with an apoptotic assay such as annexin V. However, it has been

reported that MTT can activate phosphatidylinositol-3-OH kinase [PI (3) K] and influence cell morphology in some cells, which this group suggest may ultimately affect cell viability data.²⁵⁶ As this assay measures the metabolic status of living cells and as such damaged cells may recover their viability over time, especially when cytotoxic agents are removed.

LDH assay is a measure of the release of LDH released from the lysed cell. This assay is indicative of necrotic cells as LDH is only released from cells with compromised plasma membranes as occurs in necrosis.^{160,257} These two assays were chosen for use throughout the whole of this thesis but particularly in Chapter 3 because it was thought they complemented each other when used in conjunction to measure cell viability, especially in studies investigating apoptotic and necrotic cell death pathways. Using these two viability assays, we thought that it would be possible to discern the mode of cell death. During LSI and before reperfusion morphological analysis revealed that the neonatal cardiomyocytes had ceased contracting. This was probably due to ATP depletion as a function of the hypoxic model, however, it is interesting to note that LDH release is nearly the same as control and metabolic activity is higher prior to reperfusion. Upon reperfusion metabolic activity drops and LDH release increases and even though metabolic activity is lower than during LSI, cardiomyocyte contraction is re-initiated. These observations can be explained by the findings of Gomez and colleagues.¹⁴⁸ They noticed that in MTT analysis of cardiomyocytes cells subjected to hyperkalemic solutions, there is a conservation of energy through cell membrane depolarization resulting in reduced cell viability in hypoxic conditions. They then go on to speculate this is the same protection afforded to cardiomyocytes by cardioplegic solutions. However, our data demonstrates that mitochondrial membrane potential levels in Chapter 3 have always been high in LSI in comparison to LSI/R and therefore does not agree with their statement.

Mitochondrial isolation was an essential requirement in numerous Chapters in this thesis. In Chapter 5 in order to isolate interfibrillar mitochondria (IFM), a protease nagarse, which is specifically able to isolate IFM that is located between the myofibrils, was used.¹⁹⁶ Subsarcolemmal mitochondria (SSM) are located beneath the plasma

membrane and are easily isolated by mechanical disruption of the tissue. Throughout this thesis, generally to isolate mitochondria, mechanical disruption, using a polytron processor, and a series of differential centrifugation was used. Purity of isolation was ascertained with the use of various Western blot antibodies or electron microscopy. In conjunction with other isolation protocols, a discontinuous Percoll density gradient could also be used to further separate out non-mitochondrial components such as cell membranes, myelin and other cellular debris from the isolated mitochondria. As well as using electron microscopy, proton import into mitochondria using malate dehydrogenase, measurement of $\Delta\psi_m$ and state 3 and 4 respiration rates using glutamate and malate as substrates. These methods would help to assess isolated mitochondrial integrity and function and thus confirm purity of these isolations.

8.2.5 Molecular Biology Assays

These techniques were predominantly used in Chapter 7 to prepare CyP-D for transfection. CyP-D was initially subcloned into pBluescript cloning vector which possesses many restriction enzyme site enabling CyP-D to be easily subcloned into expression vectors pcDNA3 and pcDCAG ready for transfection. All of the techniques described in Chapter 2 were all common molecular biological procedures that were performed in the laboratory of Dr Richard Heads at the Rayne institute.

Western blot was used throughout the present studies described in this thesis to measure protein expression levels. Various protein concentrations were loaded onto agarose gels and running parallel commaisse gels checked equality of protein loading. Wherever possible positive controls were also used.

8.3 Discussion of Thesis

The LSI/R model was employed in Chapter 3 to investigate the following hypotheses:

- As CyP-D binding to the ANT-VDAC complex increases with increasing oxidative stress and the integral PPIase activity induces a conformational change to form the MPTP, this may be detectable as an increase in PPIase activity or protein expression.
- As the duration of ischaemia increases, more and more cells will die by necrosis rather than apoptosis

The data presented in Chapter 3 is consistent with the second proposed hypothesis and has enabled the characterization of the LSI/R model. In assessment of the mode of cell death in LSI/R, it was observed that at lower ischaemic time points, apoptotic cells were detected in the supernatant but cell death was totally necrotic in the monolayer and supernatant. As the duration of LSI/R increased cell death increased in the supernatant and monolayer to 100% of which the cells were totally necrotic. Apoptosis was detected in 3 hour ischaemic and reperfused as well as in the 1 hour ischaemic and reperfused samples as determined by ultrastructural analysis and viability assays.

The determining factor in the severity and mode of cell death is the duration of ischaemia. This statement is in agreement with work done by other authors.²⁵⁸ They argue that apoptosis is an event which is prone to occur in every cell. However if the subroutines or controlling factors are altered then necrosis occurs. To this end, Leist gives a cogent argument in favour of this proposal, as he believes that necrosis can be represented as aborted apoptosis.²⁵⁸ A controlling factor is believed to be the severity of the insult, which in turn affects ATP energy levels. As was observed by the experiments in Chapter 3 when the severity of LSI/R was altered, although to complete the story ATP levels need to be measured in the experiments in Chapter 3. Other studies have demonstrated the effect of manipulating ATP levels which has been proven to determine the mode of cell death.^{172,259,260} During IR, apoptosis is observed in the heart that has experienced less severe insults such as in the tissue surrounding a myocardial infarction.¹⁵⁹ It has also shown here that the same insult can produce either form of cell death as has been reported elsewhere.^{172,258} The results obtained from this chapter recapitulate previous work of Taimor and co-workers who also had similar results. They used

simulated ischaemia/reperfusion to determine if cell death was predominantly necrotic or apoptotic except they used adult cardiomyocytes.¹⁶⁰ Analysis of HSP10, HSP60, HSP75 and cyclophilin revealed no difference in protein levels in comparison to their controls as assessed in primary rat neonatal cardiomyocytes. However, HSP70 protein expression increased with the progression of LSI/R reaching a peak at 3 hours LSI and 24 hour reperfusion. It is also interesting to note that HSP70 protein expression reached a maximum level at 3 hours LSI/R but decreased at 6 hours LSI/R. There may be a correlation with increasing necrotic cell death and decreasing protein expression and other previously discussed factors, but this was not explored in this Chapter.

The discovery of apoptosis in heart disease¹⁵⁹ has also shed a new light on cell death in many different pathophysiological situations, possibly offering other ways to deal with heart disease. It has been thought to be a contributory factor for heart failure following coronary artery occlusion.¹⁹⁷ The regulation and mechanisms involved in apoptosis are still not fully understood and many reports doubt the validity of current methods used to identify apoptosis.²⁶¹ The results from the electron microscopy data correlate with those of the cell viability assays, MTT and LDH. Apoptosis and necrosis has been observed in this model, but the causative factors need to be investigated. Reactive oxygen species (ROS) or free radicals are known to occur during reperfusion of ischaemic tissue.²⁶² Ideally further experimentation could involve using flow cytometry in conjunction with a ROS dye such as 2', 7' -dichlorodihydrofluorescein diacetate (H₂DCFDA), to measure ROS in this model. ROS activity may be partly responsible for the decreases in viability observed at some of the MTT and LDH assay time points. It was Bolli and colleagues who discovered that major free radical species such as O₂⁻, H₂O₂ and OONO⁻ are generated during the first few minutes of reperfusion and contribute to reperfusion injury.²⁶³ These free radical species generated upon reperfusion, exacerbate the deleterious effects of Ca²⁺ accumulation in the ischaemic mitochondrion. Recent reports state that hypoxia is known to increase ROS generation in isolated mitochondria.²⁶⁴ It is clear that calcium sequestration and production of ROS species go "hand in hand". The source of the reported ROS has been proven to be the mitochondrion^{42,221,243,265,266} which is also the target of the ROS resulting in mitochondrial damage.

This was manifested as depolarization of $\Delta\psi_m$ and eventually led to cell death.^{42,243} Using inhibitors of specific sites of the mitochondrial electron transport such as rotenone and myxothiazol may help to confirm that the source of ROS in this chapter is the mitochondrion.

This first hypothesis was proposed as it has been observed that increased oxidative stress increases binding of CyP-D to the ANT,^{140,142} coupled with the fact that CsA does not inhibit or completely inhibit binding of CyP-D to the ANT-VDAC complex.^{171,267,268} This has been observed in conditions of high Ca^{2+} where it is speculated that the pore forms without the need for CyP-D binding to the ANT.⁵⁵ Moreover, increased binding occurs as PPIase activity of CyP-D causes a conformational change in the ANT complex to form MPTP. Presumably with the increased binding there may be an increase in PPIase activity. However, in the PPIase activity experiments performed in Chapter 3 there was no statistically significant change in PPIase activity as oxidative stress increased. There were no observable differences in PPIase activity of cyclophilin between the control, LSI and LSI/R samples.

The LSI/R model used in Chapter 3 was also used to precondition rat neonatal cardiomyocytes in Chapter 4. The aim of this Chapter was:

- To investigate the role of cyclophilins and heat shock proteins in ischaemic preconditioning.

In Chapter 4 the protective role of HSP'S in delayed preconditioning was investigated. In this study HSP10, HSP60 and HSP75 protein levels remained constant in the model used. Other data from PPIase activity and protein expression indicated that cyclophilin activity and expression did not change. Instead, HSP70 expression was elevated after 1 hour simulated ischaemia (SI) and 24 hours reperfusion. This correlates with the time course needed to induce protection against LSI/R and we suggest that HSP70 may be a

contributory factor in the observed protection afforded to rat neonatal cardiomyocytes against LSI/R that was able to preserve $\Delta\psi_m$. This is not surprising as the presence of heat shock proteins are known to protect against cell death, but the protection observed may depend on the model systems.^{225,269,270} The involvement of HSP's in IPC has not been fully elucidated. They are more commonly associated with delayed preconditioning, although HSP27 and $\alpha\beta$ -crystallin have been associated with the so-called classic or early ischaemic preconditioning.²⁷¹ The application of IPC to clinical situations is impractical as described in Chapter 4. A more practical method employs the use of drugs that can mimic these effects (pharmacological preconditioning). Doxorubicin was used to pharmacologically precondition liver against IR. Nitric oxide (NO) donors can be used as pharmacological agent especially as NO has been implicated in the late phase of preconditioning.²¹⁸ As a consequence of this, they were used as described in Chapter 5 to pharmacologically precondition H9c2 embryonal rat heart derived cardiomyocyte cell line. The experiments in Chapter 5 were based on the proposal that:

1) NO induced cyclophilin and HSP70 contribute to protection against ischaemia/reperfusion injury; 2) NO donors confer protection against oxidative stress by modulating $\Delta\psi_m$.

NO donors have been used in cardiovascular therapeutics since the 19th century because they have direct vasoactive effects resulting in their usage to treat heart disease. Some of these compounds when applied to biological systems spontaneously release NO (while others need to be metabolised) that can substitute an endogenous NO limitation.²⁷² The choice of NO donor was based on previous results using SNAP (abstract from Cold Spring Harbour Meeting, 1998), as it is the donor of choice for many researchers.^{120,224,225,273-277} The proposal or hypothesis was not wholly disproved, as NO did modulate $\Delta\psi_m$ in protection against oxidative stress possibly due to the induction of HSP75. The late preconditioning effect of SNAP is comparable to doxorubin-induced protection. The mechanism was believed to involve generation of ROS, which in turn initiated a stress response.¹⁹⁴ This is similar to another report in which free radical species effect demonstrated delayed protection in a rat LAD model. The mechanism

was believed to involve upregulation of HSP's as well as antioxidants.⁹⁸ SNAP belongs to the group of NO donors known as the s-nitrosothiols (RSNOs) that require trace amounts of transition metal ion for decomposition,²⁷³ thereby yielding NO and NO⁺.^{272,278} The presence of free radicals can increase the production of NO release during hypoxia²⁷³ and in this report its toxicity was also increased. Toxicity could be due to the NO⁺, or from the reaction of NO with O₂⁻ to form H₂O₂ and OH⁻ via the Haber – Weiss reaction or peroxynitrite (OONO⁻) resulting in cell damage.¹²⁰ There are many reports concerning the cytotoxicity of NO,^{219,222,273,279,280} as well as reports demonstrating the protective effects of NO.^{220,225,277,281,282} The balance between cytotoxicity and cytoprotective activities of NO are very ill defined and depend on the concentration of exogenous NO application as well as the cell or tissue type used. Most causes of cytotoxicity reside around inhibition of mitochondrial respiration at sites I, II, IV and aconitase.^{120,127,205,280} The severity of damage was a determinant in whether cell death was by apoptotic or necrotic pathways, thereby confirming the ATP dependence on cell-mediated death. In chick embryo heart cells, SNAP resulted in s-nitrosation of caspases in the presence of *N*-Acetylcysteine (NAC), which prevents glutathione (GSH) depletion, thereby inhibiting apoptosis. However if NAC is removed, necrosis ensues.²⁸³ Low concentrations of NO may inhibit the MPTP by the action of NO⁺ stimulating Ca²⁺ efflux from mitochondria.^{61,284} NO administration has resulted in HSP production in endothelial cells, demonstrating protection against oxidative stress.²⁸¹ Xu and colleagues discovered that exogenous NO induced HSP70 in vascular smooth muscle cells via activation of the heat shock transcription factor 1 (HSF1). They provided evidence that the presence of denatured proteins as a result of NO damage initiated a stress response, activating HSF1 and thereby inducing HSP70 production.²²⁴ Kim and co-workers also determined that via a cGMP mechanism, NO induced HSP70 expressed which conferred protection against tumour necrosis factor- α (TNF- α) mediated cytotoxicity in rat hepatocytes.²²⁵ A previous report by the same group showed that upregulation of HO-1 by a low dose of NO was protective against oxidative stress. They used cycloheximide to prove this, but have not discussed the presence of other heat shock proteins in the observed delayed protection. As *de novo* HSP synthesis is a feature of delayed protection, in Chapter 4 we set out to illustrate that in the *in vitro* models using different cycles of ischaemia and reperfusion, protection against a more

lethal stress could be induced by preconditioning cells. In Chapter 6, addition of NO displayed late protection against oxidative stress. The resistance displayed in these chapters against oxidative stress was in part due to upregulation of HSP75 and maybe HSP27.

To date IPC has been demonstrated to occur in all species in which it has been tested; however the mechanisms vary from species to species and its clinical relevance has always been questioned. Yellon and co-workers demonstrated Ischaemic preconditioning in human biopsy samples.⁷⁸ Alkhulafi proved that it was possible to induce preconditioning in patients prior to coronary artery bypass grafting (CABG)²⁸⁵. There were various other studies which used human cells, biopsies to investigate IPC in humans;^{181,286} however eliciting IPC by ischaemia is not a practical method in humans. The purpose of IPC studies is to develop drugs that can be used to induce chronic resistance in humans towards myocardial ischaemia. In Chapter 6, SNAP an NO donor was successfully used to precondition rat cardiomyocyte cells. Exogenous administration of NO donors is the backbone of a diverse field of pharmacotherapeutics used in cardiovascular medicine. Lessar and co-workers using nitroglycerin, have been able to mimic late preconditioning in patients,²⁸⁷ demonstrating that as the mechanisms of IPC are elucidated then improved drugs can be developed.

In Chapter 5, subsarcolemmal (SSM) and interfibrillar (IFM) mitochondria were isolated from normal and ischaemically reperfused rats in order to evaluate:

- The $\Delta\psi_m$ in the different groups in comparison to their respective controls
- Heat shock protein expression in SSM and IFM
- Any ultrastructural differences.

In this present study on SSM and IFM, an LAD occluded rat model was used to mimic myocardial ischaemia/ reperfusion. There were no observed differences in mHSP expression between SSM and IFM in the individual groups or in respect to the controls.

Electron microscopy did not reveal any morphological differences. However, there was a statistically significant decrease in $\Delta\psi_m$ in SSM and IFM from rats subjected to 90 minutes LAD occlusion and 15 minutes reperfusion. SSM from 60 minute LAD occluded and 15 minute reperfused samples were statistically significant.

Isolation of the individual mitochondrial populations for SSM employed the use of a polytron tissue processor to homogenise tissue and differential centrifugation, which isolates SSM from the sarcolemma, just below the plasma membrane. IFM is obtained by application of protease treatment of the washed polytron pellet with Nagarse and differential centrifugation, which release the mitochondria from the myofibrils. These methods were originally devised by Palmer and colleagues¹⁹⁶ and are generally used to isolate SSM and IFM.^{200,202,206,215,249,288} These mitochondria are not identified by ultrastructural differences but by their location and biochemical properties¹⁹⁶. The differences are listed on the following page in Table 8-1.

Figure 8-1. Differences between SSM and IFM

This table demonstrates the differences between subsarcolemmal (SSM) and interfibrillar mitochondria (IFM) reported from a number of different centres. > = increase or greater.

SSM	IFM	Reference:
	> Oxidative rates	197
>HSP60 and HSP75	>Import of proteins	202,215
>Accumulation of Ca^{2+} when muscle potentiated		207
	>Aging decrease oxidative activity	198,200
	> $\Delta\psi_m$	249

In mitochondria isolated from skeletal muscle, import rates of precursor proteins were greater in IFM even though SSM had higher levels of the mitochondrial chaperone proteins HSP60, 10 and 75. The increase in protein import was attributed to the greater ATP levels in IFM. This was confirmed when ATP levels in the subfractions were equalised and the rate of protein import in both were similar. As a result of these observations this group (*Takahashi et al*) states “mitochondrial heterogeneity within a

cell type can be due to differences in the rates of protein import".²⁰² This is quite a reasonable statement as mitochondrial DNA is not able to encode all the proteins it requires so it is reliant on importing them. The same group also reported an increase in mitochondrial import of the protein malate dehydrogenase in response to increased chronic contractile activity. This resulted in an increase in mitochondrial HSP60, HSP70, and the outer membrane receptor Tom 20 levels. There was also a notable increase in cardiolipin, which along with the other factors occurred in both sub-fractions, although protein import was higher in IFM.²¹⁵ Another investigator determined that in rats subjected to endurance training, there is a differential response of skeletal muscle mitochondrial subsets in oxidative capacity that is dependent on the substrate metabolised. IFM generally possess a limited adaptability in modulating oxidative capacity. This led to the hypothesis that the less oxidative SSM lie adjacent to the plasma membrane thereby allowing a gradient of oxygen and substrate transport into the IFM lower region of the cell.²⁴⁹

Increased oxidative capacity, confirmed by increased cytochrome oxidase activity, results in a high proton motive force in IFM. Because the proton motive force generates $\Delta\psi_m$, IFM possess higher $\Delta\psi_m$ than SSM.²⁸⁸ This does not correlate with the observed $\Delta\psi_m$ in Chapter 6; SSM $\Delta\psi_m$ was lower than IFM but only after subjection to IR. It is interesting to note that in the majority of mitochondrial studies, SSM mitochondria are isolated by mechanical shearing techniques. Only enzymatic digestion releases IFM. Studies on mitochondrial physiological aspects such as aging and protein import have to consider these subpopulations. In the article by Craig and Hood, they found that ageing did not affect these parameters, but their mitochondrial isolation procedures may have released SSM only which do not demonstrate ageing affects whereas they should have isolated IFM also.²⁸⁹

Ever since cyclophilin-D (CyP-D) was confirmed to be the PPIase present in the mitochondrial matrix,^{52,290} its association with the MPTP has been suggested to be deleterious to the cell as it occurs in cell death.^{41,52} The final chapter discusses a transfection procedure, which aimed to elucidate the role of CyP-D in cell death by myocardial IR. This chapter is based on the hypothesis:

- Overexpression of CyP-D may confer protection, as cyclophilins are molecular chaperones.

The null hypothesis states that as:

- CyP-D is an important component of MPTP and as such is associated with cell death, overexpression may enhance the formation of MPTP and leads to death of the cell.

A novel transfection protocol was employed which used a novel non-viral integrin targeting peptide moiety that had been proven to possess high transfection efficiency.^{138,233,291} Green fluorescent probe (GFP) demonstrated good transfection efficiency (~50%). However, overexpression of CyP-D was not detected by Western blot, but PPIase activity assay registered a statistically significant increase as opposed to controls in all cell types tested irrespective of expression vector used. It is concluded that the transfection worked but due to the use of a non-specific antibody overexpression was not detectable by the classic means of Western blot, but CyP-D overexpression was visualised by an increase in PPIase activity by use of a specific CyP-D peptide.

Controversy exists over the components of the MPTP. Crompton and colleagues suggest that it is composed of VDAC, ANT and CyP-D, a view supported by other authors.⁵⁰ In order to verify this they constructed a fusion protein of glutathione *S*-transferase (GSH) and CyP-D. This was combined with purified VDAC and ANT to reconstitute the MPTP that on addition of Ca^{2+} and phosphate opened and although CsA blocked the pore they could not remove it once it was bound. They however were not convinced that these experiments confirmed the nature of the pore components.^{157,267} Halestrap has been unable to find evidence for the existence of VDAC. He suggests that MPTP is composed of CyP-D and ANT and as such also employed a GST/CyP-D fusion protein to investigate this. In his reconstituted pore, CyP-D bound tightly to ANT and CsA prevented binding as opposed to Crompton's

experiment in which CsA did not prevent binding. These differences in results were ascribed to the different protocols used.²⁹² It is interesting to note that under conditions of elevated Ca^{2+} CsA does not inhibit pore formation. This proves that CyP-D is not an absolute requisite for the opening of the MPTP. It is thought that *cis trans* isomerisation around a proline bond causing a conformational change that sensitises the pore to components that induce pore formation. Rather, it seems that this step can be bypassed and binding of CyP-D serves to enhance MPTP opening.¹⁴³ This is in agreement with other authors who argue about the involvement of PPIase activity in pore formation.²⁹³ Bauer and co-workers⁵⁶ screened an expression library for dominant apoptosis-inducing genes. Overexpressing ANT-1 in various cell lines resulted in spontaneous apoptosis. Overexpression of ANT-2 was perfectly innocuous. Interestingly co-transfection of CyP-D repressed apoptosis. These protective characteristics of CyP-D were evident in another report where the author demonstrated that transfection of CyP-D protected against apoptosis via a PPIase dependent mechanism.¹⁴³ This recent report by Lin and Lechleiter¹¹⁸ best describes what was attempted in Chapter 7 of this thesis. None of the vectors used were able to demonstrate overexpression by the classical method of Western blotting in Chapter 7. However, it is interesting to note that cells transfected with the CyP-D insert, irrespective of the vector used, demonstrated a significant increase in PPIase activity. Based on these results it seems probable that we may have successfully overexpressed CyP-D. However, viability assays did not demonstrate any significant protection afforded to the CyP-D transfected cells. This may indicate that although overexpression of CyP-D as determined by PPIase activity was successful, it may seem that in our model, this CyP-D insert does not afford protection in the cell models tested in Chapter 7 against lethal ischaemia and reperfusion as determined by MTT and PI viability assays. Further studies could involve the use of more flow cytometry based assays to measure differences in $\Delta\psi_m$ between CyP-D transfected and non-transfected cells subjected to lethal ischaemia and reperfusion.

8.4 Final Conclusion

This thesis has been very challenging in its attempt to define a role for cyclophilin in progression of myocardial ischaemia reperfusion injury. It has employed the use of various *in vitro* and *in vivo* models that has resulted in some interesting data, which has generated far more questions than have been answered. Most notable were the large increases in PPIase activity of the transfected cells. In all other experiments, no change in PPIase activity was observed. Cyclophilins are the subject of an emerging science. They are generally thought of as being detrimental to the cell but in some instances are regarded as survival factors.^{56,143} The studies described in this thesis focused on CyP-D, but there is emerging interest in cyclophilin A and B (CyP-A and CyP-B). CyP-A has been reported to be produced by vascular smooth muscle cell (VSMC) in response to ROS production.²⁹⁴ Other authors have implicated CyP-A in caspase activation²⁹⁵ and CyP-A and CyP-B in rheumatoid arthritis, HIV infection and inflammation.²⁹⁶ Their involvement it seems arises from their inherent PPIase activity that has been speculated to induce signalling and chemotactic response in T-cells,²⁹⁶ which is a change from its putative role as a molecular chaperone.²⁹⁷ The role of heat shock proteins was also investigated (specifically the mitochondrial chaperone complex) in order to ascertain protective functions under various conditions (as described in the previous chapters), perhaps in conjunction with cyclophilin relating to cardiac protection. Most recently research on the molecular chaperones is now investigating the role of heat shock proteins in the activation of the immune system.²⁹⁸ Indeed, interest in this field is burgeoning, as it may be relevant to many pathological conditions.

CHAPTER 9

9. LIST OF PUBLICATIONS

1. Ekaterina Monastyrskaya. **Najeem Folarin**, Igor Malyshev, Colin Green and Larisa Andreeva: Application of the nitric oxide donor SNAP to cardiomyocytes in culture provides protection against oxidative stress. Nitric Oxide.2002 Sep; 7(2): 127.

PUBLISHED ABSTRACTS

1. **Folarin N.I**, Fuller B, Andreeva L, Green C.J. Mitochondrial membrane potential expression in subsarcolemmal and interfibrillar mitochondria in myocardial ischaemia/reperfusion injury. British Transplantation Society Meeting, London (In Press) April 2003
2. **N.I.Folarin**, R.J.Heads, S.L. Hart, C.J.Green and L.V.Andreeva. CyPD overexpression in COS-7 cells. Frontiers in mitochondrial research. Albany, New York, USA.2000
3. Larisa Andreeva, **Najeem Folarin**, Roberto Moterlini and Colin Green. Differential induction of cyclophilins and heat shock proteins in cultured myocytes by nitric oxide donors. Molecular chaperones and heat shock response meeting, Cold Spring Harbor Laboratory, USA. 1998

CHAPTER 10

10. REFERENCES

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